

FISH & RICHARDSON P.C.

2200 Sand Hill Road
Suite 100
Menlo Park, California
94025

Telephone
650 322-5070

Facsimile
650 854-0875

October 2, 1998

Attorney Docket No.: 09272/006002
UC Div. of B96-021

Box Patent Application

Assistant Commissioner for Patents
Washington, DC 20231

Presented for filing is a new divisional patent application of:

Applicant: JASPER D. RINE, VICTOR L. BOYARTCHUK AND
MATTHEW N. ASHBY
Title: AFC1 AND RCE1: ISOPRENYLATED CAAX PROCESSING
ENZYMES

The prior application is assigned of record to The Regents of the University of California, a California corporation, by virtue of an assignment submitted to the Patent and Trademark Office in the prior application.

Enclosed are the following papers, including all those required to receive a filing date under 37 CFR §1.53(b):

	<u>Pages</u>
Specification	54
Claims	3
Abstract	1
Declaration	2

Enclosures:

- Associate Power of Attorney
- Postcard.

"EXPRESS MAIL" Mailing Label Number EL 079 161 347 US

Date of Deposit October 2, 1998

I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Paul H. Flores

Paul H. Flores

BOSTON

HOUSTON

NEW YORK

SOUTHERN CALIFORNIA

SILICON VALLEY

TWIN CITIES

WASHINGTON, DC

jc135 U.S. PTO
09/165460
10/02/98

BOX PATENT APPLICATION

October 2, 1998

Page 2

This application is a divisional (and claims the benefit of priority under 35 USC §120) of U.S. application serial no. 08/902,774, filed 7/30/97. The disclosure of the prior application is considered part of (and is incorporated by reference in) the disclosure of this application.

On page 1 of the specification, replace the first sentence with --This is a divisional of U.S. application serial no. 08/902,774, filed 7/30/97 (pending), which is a utility application based on U.S. provisional application serial no. 60/023,491, filed 8/7/96.--

Before calculating the fees, please cancel claims 1-9, 16 and 20-21.

This application is entitled to small entity status. Small entity status established in a previous application is still proper.

Basic filing fee	\$ 395.00
Total claims 9 in excess of 20 = 0 times \$11.00	0.00
Independent claims 2 in excess of 3 = 0 times \$41.00	0.00
Multiple dependent claims	0.00
Total filing fee:	\$ 395.00

Under 37 CFR §1.53(d), no filing fee is being paid at this time.

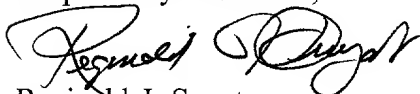
If this application is found to be INCOMPLETE, or if a telephone conference would otherwise be helpful, please call the undersigned at 650/322-5070.

Kindly acknowledge receipt of this application by returning the enclosed postcard.

Please send all correspondence to:

Reginald J. Suyat
Fish & Richardson P.C.
2200 Sand Hill Road, Suite 100
Menlo Park, CA 94025

Respectfully submitted,



Reginald J. Suyat
Reg. No. 28,172

Enclosures

98034 PAL1

APPLICATION
FOR
UNITED STATES LETTERS PATENT

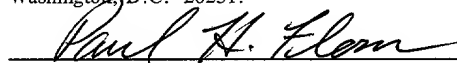
TITLE: AFC1 AND RCE1: ISOPRENYLATED CAAX PROCESSING
ENZYMES

APPLICANT: JASPER D. RINE, VICTOR L. BOYARTCHUK, MATTHEW N.
ASHBY

"EXPRESS MAIL" Mailing Label Number EL 079 161 347 US

Date of Deposit October 2, 1998

I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "**Express Mail Post Office To Addressee**" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.



Paul H. Flores

AFC1 AND RCE1: ISOPRENYLATED CAAX PROCESSING ENZYMES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority of U.S. Provisional Application Serial No.
5 60/023,491, filed August 7, 1996.

STATEMENT OF GOVERNMENT INTEREST

This invention was made with support pursuant to Grant (Contract) No.
GM 35827 awarded by the National Institutes of Health and with support pursuant to
10 Grant (Contract) Nos. 4FT-0083 and 1RT-0026 awarded by the Tobacco-Related
Disease Research Program.

BACKGROUND OF THE INVENTION

A cell possesses thousands of unique proteins that serve structural,
15 enzymatic or signaling functions. The intracellular environment is composed of a
myriad of structures and membrane-enclosed compartments. The correct subcellular
localization is critical for the proper functioning of many proteins. Proteins situated at
the lipid bilayer membrane are classified as peripheral proteins, whereas proteins
situated within the lipid bilayer membrane are classified as integral membrane proteins.
20 Integral membrane proteins possess intrinsic hydrophobic regions which are inserted into
the lipid bilayer as they are synthesized. Typically, peripheral membrane proteins are
less tightly associated with membranes and are localized to the lipid bilayer by protein-
protein interactions, by intrinsic hydrophobic properties or by the post-translational
addition of a lipid group.

25 A major class of peripheral membrane proteins, known as prenylated
proteins, are modified by isoprenoids on a so-called CAaa₁Aaa₂Xaa (CAAX) motif,
wherein C is cysteine, Aaa₁ and Aaa₂ are aliphatic amino acids and Xaa is any amino
acid. This tetra-peptide sequence is located at the proteins' carboxyl termini and
triggers a series of modification reactions. Of the approximately 30 known CAAX-
30 containing proteins, the Ras family of small GTP-binding proteins are major
constituents. Ras proteins localize at the inner surface of the plasma membrane where

they function as key components of various signal transduction pathways or participate in cytoskeletal organization and establishment of cell polarity. The critical role of the Ras proto-oncogene in controlling cell division is exemplified by the participation of mutated forms of the Ras protein in a variety of human tumors, including colorectal carcinoma, exocrine pancreatic carcinoma and myeloid leukemias. Forms of Ras in cancer cells have mutations that distinguish the protein from Ras in normal cells.

The presence of the CAaa₁Aaa₂Xaa motif sequence targets the protein for at least 3 post-translational modifications. Generally, such modifications include prenylation of the cysteine amino acid, proteolytic removal of the terminal three amino acids (*i.e.*, the Aaa₁Aaa₂Xaa tripeptide) and methylesterification of the prenylated cysteine, *i.e.*, the C-terminus. More particularly, in the first step, a 15 carbon farnesyl or a 20 carbon geranylgeranyl isoprenyl lipid is added to the cysteine residue. The lipid which is added depends upon the amino acid at the "X" position. Following prenylation, the terminal tripeptide, *i.e.*, the Aaa₁Aaa₂Xaa tripeptide, is removed by a membrane-bound endoprotease. Thereafter, the resulting C-terminal isoprenylated cysteine is methylesterified.

It has been determined that prenylation of the CAAX motif is essential for the proper functioning of every prenylated protein that has been tested to date. However, the functional requirement of CAAX proteolysis has not been rigorously evaluated because the gene encoding the protease has been elusive. This is true despite the fact that the entire yeast genome has been sequenced and the sequences deposited in GenBank. Unfortunately, elucidation of the complete yeast genome in the absence of functional information for each yeast gene is insufficient for identification of any particular gene. Although many open reading frames (ORFs) have been identified, it is not known whether these ORFs encode functional mRNAs.

Kato, *et al.* (*Proc. Natl. Acad. Sci. USA*, 89:9554-9558 (1992)) monitored foci formation of NIH3T3 cells transformed with activated forms of Ras with altered CAAX sequences. They found that one sequence, CVYS, when substituted for the normal Ras CAAX sequence appeared not to have undergone proteolysis and resulted in approximately 50% reduction in foci formation. Unfortunately, the design of this experiment was not ideal because it relied on the heterologous expression of Ras

from an SV40 promoter, which resulted in a considerably higher expression level than the physiological Ras promoter. Moreover, the CAAX sequence CVYS displayed a prenylation defect.

In view of the foregoing, there remains a need in the art for the identification of the genes encoding the polypeptides that participate in the post-prenylation modification reactions so that the functional importance of such enzymes can be elucidated.

SUMMARY OF THE INVENTION

The present invention includes the discovery of two families of genes which encode polypeptides that mediate the proteolytic removal of an AAX tripeptide from a prenylated CAAX protein in a cell. In yeast, the families of genes are represented by the genes *AFC1* and *RCE1* which encode the polypeptides Afc1p and Rce1p, respectively.

Accordingly, the invention provides vectors that includes a nucleic acid sequence which encodes an Afc1p or Rce1p polypeptide (or both polypeptides), or conservatively modified variations of Afc1p or Rce1p. Exemplar nucleic acids which encode Afc1p, or Rce1p include those set forth in SEQ ID NO:1 and SEQ ID NO:2. Recombinant cells, including recombinant yeast cells, which comprise a vector nucleic acid of the invention are also provided.

In one class of embodiments, the vector of the invention provides a nucleic acid sequence which hybridizes under stringent conditions to a nucleic acid selected from the group consisting of the *AFC1*, and *RCE1* genes. Exemplar nucleic acids with the desired hybridization properties include those represented by the sequences of SEQ ID NO:1 and SEQ ID NO:2.

The invention provides isolated polypeptides, such as Afc1p and Rce1p, encoded by the vectors of the invention. Exemplar polypeptides include those represented by SEQ ID NO:3 and SEQ ID NO:4. Antibodies which specifically bind to the polypeptides of the invention are also provided.

In addition to nucleic acids, cells, polypeptides and antibodies, a variety of useful methods and assays are provided by the present invention. In one

embodiment, the invention provides methods for inhibiting the proteolytic removal of an AAX tripeptide from a prenylated CAAX protein in a cell. Exemplar prenylated CAAX proteins include the Ras protein, α -factor, and the γ -subunit of the heterotrimeric G-protein. In these methods, a mutation is introduced into an *AFC1* and/or *RCE1* gene.

5 In one class of embodiments, the invention provides methods for inhibiting the proteolytic removal of an AAX tripeptide from a prenylated CAAX protein in a cell. In this class of embodiments, the activity of the Afc1p or Rce1p protein is blocked using an inhibitor. Exemplar inhibitors include 1,10-phenanthroline and NME 181.

10 The invention provides assays for testing the inhibitory activity of a potential inhibitor of the Afc1p or Rce1p proteases, which are responsible for the proteolytic removal of an AAX tripeptide of a CAAX protein in a cell. In the assay method, a test compound to be tested for inhibitory activity is provided. The test compound is contacted to a cell expressing either the *AFC1* or *RCE1* genes, or both.
15 The transcriptional or translational activity of the genes or, alternatively, the activity of the encoded proteins, is measured, and typically compared to a reference, such as a control assay which establishes the activity of the measured activity in the absence of the test compound. One convenient activity which is mediated by the *AFC1* and *RCE1* genes is heat shock sensitivity of cells. Accordingly, in one embodiment, the measured activity is heat shock sensitivity. In a second convenient assay, the level of Afc1p or Rce1p protein in a population of cells is measured in a standard immunological assay, such as an ELISA.

20 In addition, the present invention provides an improved method for monitoring heat shock sensitivity, particularly in yeast, is provided. In this method, a plurality of aliquoted yeast strains in liquid are provided. Each strain is separated into a test population of cells and a control population of cells. The test population of cells is heated to a heat shock temperature of between about 40°C and about 60°C for a time period of between about 30 seconds and about 10 minutes, followed by cooling to a temperature of between about 0°C and about 35°C. In a preferred embodiment, the test
25 population of cells is heated and cooled in a PCR thermocycler to allow for better temperature control. The test population of cells and the control population of cells are

grown on growth media and quantitated. The number of test and control cells are compared. The comparison of the number of cells in the test population and the control population provides a measure of heat shock sensitivity. Exemplar yeast strains include $\Delta afc1$, $\Delta rce1$, and the double deletion strain $\Delta afc1\text{-}\Delta rce1$.

5

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton et al. (1994) *Dictionary of Microbiology and Molecular Biology*, second edition, John Wiley and Sons (New York); Walker (ed) (1988) *The Cambridge Dictionary of Science and Technology*, The press syndicate of the University of Cambridge, NY; and Hale and Marham (1991) *The Harper Collins Dictionary of Biology* Harper Perennial, NY provide one of skill with a general dictionary of many of the terms used in reference to this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, certain preferred methods and materials are described in detail. For purposes of the present invention, the following terms are defined below.

"CAaa₁Aaa₂Xaa" or, interchangeably, "CAAX," as used herein, refers to a carboxy-terminal motif sequence, wherein C is cysteine, Aaa₁ and Aaa₂ are aliphatic amino acids and Xaa is any one of a number of different amino acids. The presence of the CAaa₁Aaa₂Xaa motif sequence targets the protein for at least 3 post-translational modifications. Such modifications include prenylation of the cysteine amino acid, proteolytic removal of the terminal three amino acids (*i.e.*, the Aaa₁Aaa₂Xaa tripeptide) and methylesterification of the prenylated cysteine, *i.e.*, the C-terminus. Examples of CAAX-containing proteins include, but are not limited to, fungal mating pheromones, RAS proteins, nuclear lamins and the γ -subunit of trimeric G-proteins (*see, e.g.*, Hrycyna, *et al.*, *EMBO Journal*, 10(7):1699-1709 (1991)).

An "Aaa₁Aaa₂Xaa tripeptide," as used herein refers to the terminal three amino acids of the CAaa₁Aaa₂Xaa motif sequence.

A "prenylated CAaa₁Aaa₂Xaa protein" refers to a protein containing a CAaa₁Aaa₂Xaa motif sequence, wherein the cysteine amino acid has been prenylated by the addition of a geranyl, farnesyl or geranylgeranyl lipid.

"Proteolytic cleavage," as used herein, refers to the removal of the terminal three amino acids of the CAaa₁Aaa₂Xaa motif sequence through the cleavage of a peptide bond by a protease.

"Inhibit" or, interchangeably, "antagonize," or "blocking the activity" as used herein, refers to the reduction or prevention of a reaction or process.

A "yeast strain" is a population of yeast cells, each of which share a particular phenotype or a particular genotype.

A "test population of cells" is a population of cells to be characterized, *e.g.*, in a method or an assay. A "control" population of cells is a population of cells which are used to determine that an observed effect in a method or assay is the result of experimental manipulation, and not the result of an unknown or unintended environmental parameter.

A "vector" is a composition which can transduce, transfect, transform or infect a cell, thereby causing the cell to replicate or express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. A cell is "transduced" by a nucleic acid when the nucleic acid is translocated into the cell from the extracellular environment. Any method of transferring a nucleic acid into the cell may be used; the term, unless otherwise indicated, does not imply any particular method of delivering a nucleic acid into a cell, nor that any particular cell type is the subject of transduction. A cell is "transformed" by a nucleic acid when the nucleic acid is transduced into the cell and stably replicated. A vector includes a nucleic acid (ordinarily RNA or DNA) to be expressed by the cell. This nucleic acid is optionally referred to as a "vector nucleic acid." A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. A "cell transduction vector" is a vector which encodes a nucleic acid which is expressed in a cell once the nucleic acid is transduced into the cell.

A "promoter" is an array of nucleic acid control sequences which direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter which is active under most environmental and developmental conditions. An "inducible" promoter is a promoter which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism, but not in other tissue types from the same organism.

The term "operably linked" refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence optionally includes the complementary sequence thereof.

The term "subsequence" in the context of a particular nucleic acid sequence refers to a region of the nucleic acid equal to or smaller than the specified nucleic acid. A "recombinant nucleic acid" comprises or is encoded by one or more nucleic acids that are derived from a nucleic acid which was artificially constructed. For example, the nucleic acid can comprise or be encoded by a cloned nucleic acid formed by joining heterologous nucleic acids as taught, *e.g.*, in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger) and in Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3 (Sambrook). Alternatively, the nucleic acid can be synthesized chemically. The term "recombinant" when used with reference to a cell indicates that the cell replicates or expresses a nucleic acid, or expresses a peptide or protein encoded by a nucleic acid whose origin is exogenous to the cell.

Recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also express genes found in the native form of the cell wherein the genes are re-introduced into the cell or a progenitor of the cell by artificial means.

5 The terms "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state.

10 "Stringent hybridization" and "Stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* part I chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, highly
15 stringent hybridization and wash conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

20 An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin per 50 mL at 42°C, with the hybridization being carried out overnight. An example of stringent wash conditions is a .2x SSC wash at 65°C for 15 minutes (*see*, Sambrook, *supra* for a
25 description of SSC buffer). Often the high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 4x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 2x
30 (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

5 The term "identical" in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. When percentage of sequence identity is used in reference to proteins or peptides it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid
10 residues are substituted for other amino acid residues with similar chemical properties (*e.g.* charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the
15 art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, *e.g.*, according to known
20 algorithm. *See, e.g.*, Meyers and Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988); Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482; Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443; Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444; Higgins and Sharp (1988) *Gene*, 73: 237-244 and Higgins and Sharp (1989) *CABIOS* 5: 151-153; Corpet, *et al.* (1988) *Nucleic Acids Research* 16, 10881-90;
25 Huang, *et al.* (1992) *Computer Applications in the Biosciences* 8, 155-65, and Pearson, *et al.* (1994) *Methods in Molecular Biology* 24, 307-31. Alignment is also often performed by inspection and manual alignment.

 "Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acids which encode identical or essentially identical amino acid
30 sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large

number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence. Furthermore, one of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservatively modified variations" where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplar immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist, for example, as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)₂, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)₂ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the F(ab)₂ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see, Fundamental Immunology*, Third Edition, W.E. Paul, ed., Raven Press, N.Y. (1993), which is incorporated herein by reference, for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies. Antibodies in the context of the present invention are optionally derived from libraries of recombinant antibodies in phage or similar vectors (*see, e.g., Huse et al. (1989) Science 246: 1275-1281; and Ward, et al. (1989) Nature 341: 544-546; and Vaughan et al. (1996) Nature Biotechnology, 14: 309-314*).

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

Proteins with a CAAX motif, such as Ras, a-factor and the γ -subunit of heterotrimeric G-protein, are post-translationally modified by prenylation of the cysteine, proteolytic removal of the terminal three residues and methylation-esterification of the newly-formed carboxyl group. A novel, farnesylation-dependent

endoproteolytic activity named *RACE* (Ras and a-factor Converting Enzyme) was previously discovered. Numerous efforts to identify a CAAX protease gene responsible for the activity were unsuccessful. An autocrine arrest, sensitized selection for such mutants was developed. It involved ectopic expression of the a-factor receptor in a cells and utilized a CAAX sequence permutation defective for proteolysis. 127 mutants were isolated and characterized. 24 of these mutants had altered substrate specificity, of which 2 had novel alleles of *RAM1*. The remaining 22 had mutations in a single new gene, *AFC1* (a-Factor Convertase).

Afc1p is the first identified farnesylation-dependent zinc metalloprotease. It is an integral membrane protein localized to internal membranes. Mutations in the HEXXH motif found in the protein, and characteristic of zinc metalloproteases, destroy Afc1p function. Null alleles of *AFC1* are viable and produce lowered levels of mature a-factor. The residual pheromone produced by these cells implies the existence of multiple prenyl-dependent proteases. Ras2p with a proteolysis defective C-terminus has altered biological properties, suggesting that CAAX proteolysis is important for Ras function. Accordingly, *AFC1* and the proteins encoded by the protein are attractive targets for therapeutics against Ras dependent cancers. Because of this, assays which can be used to detect the inhibition of *AFC1* activity are of immediate commercial value to pharmaceutical companies.

RCE1 encodes a 315 amino acids long protein. Extensive searches against public molecular biology databases revealed no significant homologies to any known gene. Conceptual translation of the sequence obtained by mouse cDNA sequencing project (XREF Clone ID 331228, GenBank accession W14344, NCBI ID 521315) shows 46% identity on 49 aa stretch (63% positives) to Rce1p. The Rce1 protein sequence has limited similarity to sequence blocks characteristic of signal peptidases type II (SPase II), class A8 (lipoprotein signal peptidase, which recognize a conserved sequence and cuts in front of a cysteine to which a glyceride-fatty acid lipid is attached). Even though there is limited similarity, a consensus sequence for A8 SPase II is not present. Analysis of the Rce1 protein sequence predicts the presence of a number of transmembrane domains, suggesting that Rce1p is an integral membrane protein.

A deletion of *RCE1* in haploid yeast cells of a-mating type has no effect on viability; however, it results in reduced a-factor halo size. Protease assays (see, Ashby and Rine "Ras and a- Factor Converting Enzyme" (1995) *Methods in Enzymology* 230:235) detect reduced proteolytic activity in the membrane preparations from the *RCE1* null strain. *RCE1* deletion, combined with a deletion of *AFC1*, causes complete sterility of a cells due to a complete bloc in proteolytic processing of a-factor.

RCE1 seems to affect activity of yeast homologues of Ras oncoproteins. When deletion of *RCE1* is combined with temperature-sensitive alleles of *RAS2*, it further decreases the viability of the yeast cells at elevated temperatures. The same deletion decreases the heat shock sensitivity of the yeast strains carrying an activated form of Ras protein *RAS^{val19}*. This allele is analogous to the mutation in the mammalian *Ras* - *Ras^{val12}* found in a number of cancers. Yeast strains carrying the deletion also show increased intracellular localization of *Ras2p* (presumably to internal membranes) as measured by completely functional *GFP-Ras2* fusions. All of this shows that *RCE1* modulates activity of the yeast homologue of Ras oncoproteins, without completely inhibiting its activity. *RCE1*, therefore, is an attractive target for pharmaceutical treatments directed on reduction of elevated Ras activity, found in a many malignancies. Because of this, assays which can be used to detect the inhibition of *RCE1* activity are of immediate commercial value to pharmaceutical companies for the identification of therapeutic compounds against Ras-mediated cancers.

Vectors, Cloning, Nucleic Acids and Proteins

The vectors of the invention include a vector nucleic acid, and optionally include components for packaging the vector nucleic acid to facilitate entry of the nucleic acid into a cell. The vector nucleic acid includes a nucleic acid subsequence which encodes a nucleic acid or protein of the invention. The subsequence is typically cloned into a cloning site in the vector nucleic acid which is designed to facilitate recombinant manipulation. A variety of commercially or commonly available vectors and vector nucleic acids can be converted into a vector of the invention by cloning a nucleic acid encoding a protein of the invention into the commercially or commonly available vector. A variety of common vectors suitable for this purpose are well known

in the art. For cloning in bacteria, common vectors include pBR322 derived vectors such as pBLUESCRIPT™, and λ-phage derived vectors. In yeast, vectors include Yeast Integrating plasmids (*e.g.*, YIp5) and Yeast Replicating plasmids (the YRp series plasmids), Yeast Centromeric plasmids (the YCp series of plasmids) and pGPD-2.

5 Expression in mammalian cells can be achieved using a variety of plasmids, including pSV2, pBC12BI, and p91023, as well as lytic virus vectors (*e.g.*, vaccinia virus, adeno virus, and baculovirus), episomal virus vectors (*e.g.*, bovine papillomavirus), and retroviral vectors (*e.g.*, murine retroviruses).

10 The nucleic acid sequence encoding a selected polypeptide is placed under the control of a promoter. A extremely wide variety of promoters are well known, and can be used in the vectors of the invention, depending on the particular application.

Ordinarily, the promoter selected depends upon the cell in which the promoter is to be active. Other expression control sequences such as ribosome binding sites, transcription termination sites and the like are optionally included. For *E. coli*, example control

15 sequences include the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences typically include a promoter which optionally includes an enhancer derived from

immunoglobulin genes, SV40, cytomegalovirus, *etc.*, and a polyadenylation sequence, and may include splice donor and acceptor sequences. In yeast, convenient promoters

20 include GAL1,10 (Johnson and Davies (1984) *Mol. Cell. Biol.* 4:1440-1448) ADH2 (Russell *et al.* (1983) *J. Biol. Chem.* 258:2674-2682), PH05 (*EMBO J.* (1982) 6:675-680), and MFα1 (Herskowitz and Oshima (1982) in *The Molecular Biology of the Yeast Saccharomyces* (eds. Strathern, Jones, and Broach) Cold Spring Harbor Lab., Cold Spring Harbor, N.Y., pp. 181-209).

25 Multicopy plasmids with selective markers, such as *LEU2*, *URA3*, *TRP1*, and *HIS3* is also commonly used. A number of yeast expression plasmids such as YEp6, YEp13, YEp4 can be used as expression vectors. A gene of interest can be fused, *e.g.*, to any of the promoters in known yeast vectors.

30 The above-mentioned plasmids have been fully described in the literature (Botstein *et al.* (1979) *Gene* 8:17-24; Broach, *et al.* (1979) *Gene*, 8:121-133). For a discussion of yeast expression plasmids, *see, e.g.*, Parents, B., *YEAST* (1985), and Ausbel, Sambrook and Berger, *all supra*).

Given the strategy for making the vectors and nucleic acids of the present invention, one of skill can construct a variety of vectors and nucleic acid clones containing functionally equivalent nucleic acids. Cloning methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, CA, and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

The nucleic acids provided by this invention, whether RNA, cDNA, genomic DNA, or a hybrid of the various combinations, are isolated from biological sources or synthesized *in vitro*. The nucleic acids and vectors of the invention are present in transformed or transfected whole cells, in transformed or transfected cell lysates, or in a partially purified or substantially pure form.

In vitro amplification techniques suitable for amplifying sequences to provide a nucleic acid, or for subsequent analysis, sequencing or subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Q β -replicase amplification and other RNA polymerase mediated

techniques (e.g., NASBA) are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3, 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35, 1826; Landegren *et al.*, (1988) *Science* 241, 1077-1080; Van Brunt (1990) *Biotechnology* 8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560; Barringer *et al.* (1990) *Gene* 89, 117, and Sooknanan and Malek (1995) *Biotechnology* 13: 563-564.

Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids are summarized in Cheng *et al.* (1994) *Nature* 369: 684-685 and the references therein. One of skill will appreciate that essentially any RNA can be converted into a double stranded DNA suitable for restriction digestion, PCR expansion and sequencing using reverse transcriptase and a polymerase. *See*, Ausbel, Sambrook and Berger, *all supra*.

Oligonucleotides for *in vitro* amplification methods or for use as gene probes, for example, are typically chemically synthesized according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), *Tetrahedron Letts.*, 22(20):1859-1862, e.g., using an automated synthesizer, as described in Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.*, 12:6159-6168. Purification of oligonucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier (1983) *J. Chrom.* 255:137-149. The sequence of the synthetic oligonucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology* 65:499-560.

The polypeptides of the invention can be synthetically prepared in a wide variety of well-know ways. For instance, polypeptides of relatively short length can be synthesized in solution or on a solid support in accordance with conventional techniques. *See*, e.g., Merrifield (1963) *J. Am. Chem. Soc.* 85:2149-2154. Various automatic synthesizers are commercially available and can be used in accordance with known

protocols. See, e.g., Stewart and Young (1984) *Solid Phase Peptide Synthesis*, 2d. ed., Pierce Chemical Co. As described in more detail herein, the polypeptide of the invention are most preferably made using recombinant techniques, by expressing the polypeptides in host cells and purifying the expressed proteins.

5 In a preferred embodiment, the polypeptides, or subsequences thereof, are synthesized using recombinant DNA methodology. Generally this involves creating a DNA sequence that encodes the protein, through recombinant, synthetic, or *in vitro* amplification techniques, placing the DNA in an expression cassette under the control of a particular promoter, expressing the protein in a host cell, isolating the expressed
10 protein and, if required, renaturing the protein.

Once a nucleic acid encoding a polypeptide of the invention is isolated and cloned, the nucleic acid is optionally expressed in recombinantly engineered cells known to those of skill in the art. Examples of such cells include bacteria, yeast, plant, filamentous fungi, insect (especially employing baculoviral vectors), and mammalian
15 cells. The recombinant nucleic acids are operably linked to appropriate control sequences for expression in the selected host. For *E. coli*, example control sequences include the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences typically include a promoter and preferably an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus, *etc.*, and a polyadenylation sequence, and optionally include
20 splice donor and acceptor sequences.

Plasmids of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Yeast transformation is
25 conveniently performed by one of two common procedures. In one procedure, yeast cells are first converted into protoplasts using zymolyase, lyticase or glusulase, followed by addition of DNA and polyethylene glycol (PEG). The PEG-treated protoplasts are then regenerated in a 3% agar medium under selective conditions. Details of this procedure are given in Beggs (1978) *Nature* (London) 275:104-109, and Hinnen, *et al.*
30 (1978) *Proc. Natl. Acad. Sci. USA* 75:1929-1933. The second procedure does not involve removal of the cell wall. Instead, the cells are treated, e.g., with lithium

chloride or acetate and PEG and put on selective plates (Ito, *et al.* (1983) *J. Bact.* 153:163-168).

Cells transformed by plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the amp, gpt, neo and hyg genes.

5 Viral vectors of the invention transduce nucleic acids into cells within the host range of the viral vector.

Once expressed, the recombinant polypeptides can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see, generally, R. Scopes, Polypeptide Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in Enzymology Vol. 182: Guide to Polypeptide Purification.*, Academic Press, Inc. N.Y. (1990)). Once purified, partially or to homogeneity as desired, the polypeptides may then be used (*e.g.*, as immunogens for antibody production).

After chemical synthesis, biological expression or purification, the
15 polypeptide(s) may possess a conformation substantially different than the native conformations of the constituent polypeptides. In this case, it is helpful to denature and reduce the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Methods of reducing and denaturing polypeptides and inducing re-folding are well known to those of skill in the art (See, Debinski *et al.* (1993) *J. Biol. Chem.*, 268: 14065-14070; Kreitman and Pastan (1993) *Bioconjug. Chem.*, 4: 581-585; and
20 Buchner, *et al.*, (1992) *Anal. Biochem.*, 205: 263-270). Debinski *et al.*, for example, describe the denaturation and reduction of inclusion body polypeptides in guanidine-DTE. The polypeptide is then refolded in a redox buffer containing oxidized glutathione and L-arginine.

25 One of skill will recognize that modifications can be made to the polypeptides without diminishing their biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion polypeptide. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation
30 site, or additional amino acids (*e.g.*, poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

Making Conservative Modifications of the Nucleic Acids and Polypeptides of the Invention.

One of skill will appreciate that many conservative variations of the polypeptides and vectors disclosed yield essentially identical polypeptides and vectors.

5 For example, due to the degeneracy of the genetic code, "silent substitutions" (*i.e.*, substitutions of a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of every nucleic acid sequence which encodes an amino acid. Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence are substituted with different amino acids
10 with highly similar properties (*see*, the definitions section, *supra*), are also readily identified as being highly similar to a disclosed amino acid sequence, or to a disclosed nucleic acid sequence which encodes an amino acid. Such conservatively substituted variations of each disclosed sequence are a feature of the present invention.

One of skill will recognize many ways of generating alterations
15 (introducing mutations) in a given nucleic acid sequence. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques. *See*, Gilman and
20 Smith (1979) *Gene* 8:81-97; Roberts *et al.* (1987) *Nature* 328:731-734 and Sambrook, Innis, Ausbel, Berger, Needham VanDevanter and Mullis (*all supra*).

Most commonly, amino acid sequences are altered by altering the corresponding nucleic acid sequence and expressing the polypeptide. However, polypeptide sequences are also optionally generated synthetically on commercially
25 available peptide synthesizers to produce any desired polypeptide (*see*, Merrifield, and Stewart and Young, *supra*). General knowledge regarding the nature of proteins and nucleic acids allows one of skill to select appropriate sequences with activity similar or equivalent to the nucleic acids, vectors and polypeptides disclosed herein. The definitions section herein describes exemplar conservative amino acid substitutions.

30 Most modifications to nucleic acids and polypeptides are evaluated by routine screening techniques in suitable assays for the desired characteristic. For

instance, changes in the immunological character of a polypeptide can be detected by an appropriate immunological assay. Modifications of other properties such as nucleic acid hybridization to a target nucleic acid, redox or thermal stability of a protein, hydrophobicity, susceptibility to proteolysis, or the tendency to aggregate are all assayed
5 according to standard techniques.

Human Homologues of *AFCI* and *RCEI*

The present invention provides for mammalian homologues to the yeast *AFCI* and *RCEI* genes. A Genbank search for nucleic acids encoding sequences similar
10 to the proteins encoded by the respective genes revealed expression sequence tags (ESTs) with homology to the yeast genes. The entries are found at accession number z43273 (a partial cDNA encoding a human protein with similarity to *AFCI*) and w14344 (a partial cDNA encoding a mouse protein with similarity to *RCEI*).

Complete mammalian homologues to *AFCI* and *RCEI* are isolated in a
15 variety of ways. In one embodiment, the *Afc1p* or *Rce1p* polypeptides, or polypeptides encoded by the identified mammalian sequences, are used to raise antibodies as described herein. The antibodies are used to screen expression libraries for polypeptides with homology to the immunogen used to raise the antibody. Thus, the invention provides an isolated clone encoding a polypeptide which binds to an antibody encoded
20 by a mammalian *AFCI* or *RCEI* homologue.

In another embodiment, the nucleic acids encoded by the yeast *AFCI* or *RCEI* genes, or mammalian nucleic acids encoded by the GenBank nos z43273 or w14344 are labeled and hybridized to a mammalian cDNA or genomic DNA library under increasingly stringent conditions. Clones which hybridize under moderate to
25 stringent conditions are homologous to the probe sequences. Preferred clones hybridize to the selected labeled nucleic acid under stringent conditions. Mammalian cDNA and genomic libraries are widely available, and methods of hybridizing nucleic acids to the libraries are well known.

In yet another embodiment, the invention provides PCR probes which are
30 used to amplify a mammalian *AFCI* or *RCEI* homologue from a library or tissue sample. Most typically, amplification primers are between 8 and 100 nucleotides in

length, and preferably between about 10 and 30 nucleotides in length. More typically, the primers are between about 15 and 25 nucleic acids in length. One of skill will recognize that the 3' end of an amplification primer is more important for PCR than the 5' end. Investigators have reported PCR products where only a few nucleotides at the 3' end of an amplification primer were complementary to a DNA to be amplified. In this regard, nucleotides at the 5' end of a primer can incorporate structural features unrelated to the target nucleic acid; for instance, in one embodiment, a sequencing primer hybridization site (or a complement to such as primer, depending on the application) is incorporated into the amplification primer, where the sequencing primer is derived from a primer used in a standard sequencing kit, such as one using a biotinylated or dye-labeled universal M13 or SP6 primer. Alternatively, the primers optionally incorporate restriction endonuclease sites. The primers are selected so that there is no complementarity between any known sequence which is likely to occur in the sample to be amplified and any constant primer region. One of skill will appreciate that constant regions in primer sequences are optional.

Typically, all primer sequences are selected to hybridize only to a perfectly complementary DNA, with the nearest mismatch hybridization possibility from known DNA sequences which are likely to occur in the sample to be amplified having at least about 50 to 70% hybridization mismatches to sequences which are known to be in the sample and which do not encode a nucleic acid of the invention, and preferably 100% mismatches for the terminal 5 nucleotides at the 3' end of the primer. Alternatively, a series of degenerate primers with universal base acceptors at ambiguous codon positions are used in parallel reactions to amplify a nucleic acid.

The primers are selected so that no secondary structure forms within the primer. Self-complementary primers have poor hybridization properties, because the complementary portions of the primers self hybridize (*i.e.*, form hairpin structures). The primers are also selected so that the primers do not hybridize to each other, thereby preventing duplex formation of the primers in solution, and possible concatenation of the primers during PCR. If there is more than one constant region in the primer, the constant regions of the primer are selected so that they do not self-hybridize or form hairpin structures.

Where sets of amplification primers (*i.e.*, the 5' and 3' primers used for exponential amplification) are of a single length, the primers are selected so that they have roughly the same, and preferably exactly the same overall base composition (*i.e.*, the same A+T to G+C ratio of nucleic acids). Where the primers are of differing
5 lengths, the A+T to G+C ratio is determined by selecting a thermal melting temperature for the primer-DNA hybridization, and selecting an A+T to G+C ratio and probe length for each primer which has approximately the selected thermal melting temperature.

One of skill will recognize that there are a variety of possible ways of
10 performing the above selection steps, and that variations on the steps are appropriate. Most typically, selection steps are performed using simple computer programs to perform the selection as outlined above; however, all of the steps are optionally performed manually. One available computer program for primer selection is the MacVector program from Kodak. In addition to commercially available programs for
15 primer selection, one of skill can easily design simple programs for any of the preferred selection steps. Amplification primers can be selected to provide amplification products that span specific deletions, truncations, and insertions in an amplification target, thereby facilitating the detection of specific abnormalities such as a transposon insertion as described herein.

20

Antibodies to Afc1p and to Rce1p

Antibodies are raised to Afc1p and Rce1p polypeptides of the present invention, including individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms.
25 Additionally, antibodies are raised to these polypeptides in either their native configurations or in non-native configurations. Anti-idiotypic antibodies can also be generated. Many methods of making antibodies are known to persons of skill. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods
30 are known.

instance, changes in the immunological character of a polypeptide can be detected by an appropriate immunological assay. Modifications of other properties such as nucleic acid hybridization to a target nucleic acid, redox or thermal stability of a protein, hydrophobicity, susceptibility to proteolysis, or the tendency to aggregate are all assayed according to standard techniques.

Human Homologues of *AFC1* and *RCE1*

The present invention provides for mammalian homologues to the yeast *AFC1* and *RCE1* genes. A Genbank search for nucleic acids encoding sequences similar to the proteins encoded by the respective genes revealed expression sequence tags (ESTs) with homology to the yeast genes. The entries are found at accession number z43273 (a partial cDNA encoding a human protein with similarity to *AFC1*) and w14344 (a partial cDNA encoding a mouse protein with similarity to *RCE1*).

Complete mammalian homologues to *AFC1* and *RCE1* are isolated in a variety of ways. In one embodiment, the *Afc1p* or *Rce1p* polypeptides, or polypeptides encoded by the identified mammalian sequences, are used to raise antibodies as described herein. The antibodies are used to screen expression libraries for polypeptides with homology to the immunogen used to raise the antibody. Thus, the invention provides an isolated clone encoding a polypeptide which binds to an antibody encoded by a mammalian *AFC1* or *RCE1* homologue.

In another embodiment, the nucleic acids encoded by the yeast *AFC1* or *RCE1* genes, or mammalian nucleic acids encoded by the GenBank nos z43273 or w14344 are labeled and hybridized to a mammalian cDNA or genomic DNA library under increasingly stringent conditions. Clones which hybridize under moderate to stringent conditions are homologous to the probe sequences. Preferred clones hybridize to the selected labeled nucleic acid under stringent conditions. Mammalian cDNA and genomic libraries are widely available, and methods of hybridizing nucleic acids to the libraries are well known.

In yet another embodiment, the invention provides PCR probes which are used to amplify a mammalian *AFC1* or *RCE1* homologue from a library or tissue sample. Most typically, amplification primers are between 8 and 100 nucleotides in

length, and preferably between about 10 and 30 nucleotides in length. More typically, the primers are between about 15 and 25 nucleic acids in length. One of skill will recognize that the 3' end of an amplification primer is more important for PCR than the 5' end. Investigators have reported PCR products where only a few nucleotides at the 3' end of an amplification primer were complementary to a DNA to be amplified. In this regard, nucleotides at the 5' end of a primer can incorporate structural features unrelated to the target nucleic acid; for instance, in one embodiment, a sequencing primer hybridization site (or a complement to such as primer, depending on the application) is incorporated into the amplification primer, where the sequencing primer is derived from a primer used in a standard sequencing kit, such as one using a biotinylated or dye-labeled universal M13 or SP6 primer. Alternatively, the primers optionally incorporate restriction endonuclease sites. The primers are selected so that there is no complementarity between any known sequence which is likely to occur in the sample to be amplified and any constant primer region. One of skill will appreciate that constant regions in primer sequences are optional.

Typically, all primer sequences are selected to hybridize only to a perfectly complementary DNA, with the nearest mismatch hybridization possibility from known DNA sequences which are likely to occur in the sample to be amplified having at least about 50 to 70% hybridization mismatches to sequences which are known to be in the sample and which do not encode a nucleic acid of the invention, and preferably 100% mismatches for the terminal 5 nucleotides at the 3' end of the primer. Alternatively, a series of degenerate primers with universal base acceptors at ambiguous codon positions are used in parallel reactions to amplify a nucleic acid.

The primers are selected so that no secondary structure forms within the primer. Self-complementary primers have poor hybridization properties, because the complementary portions of the primers self hybridize (*i.e.*, form hairpin structures). The primers are also selected so that the primers do not hybridize to each other, thereby preventing duplex formation of the primers in solution, and possible concatenation of the primers during PCR. If there is more than one constant region in the primer, the constant regions of the primer are selected so that they do not self-hybridize or form hairpin structures.

Where sets of amplification primers (*i.e.*, the 5' and 3' primers used for exponential amplification) are of a single length, the primers are selected so that they have roughly the same, and preferably exactly the same overall base composition (*i.e.*, the same A+T to G+C ratio of nucleic acids). Where the primers are of differing
5 lengths, the A+T to G+C ratio is determined by selecting a thermal melting temperature for the primer-DNA hybridization, and selecting an A+T to G+C ratio and probe length for each primer which has approximately the selected thermal melting temperature.

One of skill will recognize that there are a variety of possible ways of
10 performing the above selection steps, and that variations on the steps are appropriate. Most typically, selection steps are performed using simple computer programs to perform the selection as outlined above; however, all of the steps are optionally performed manually. One available computer program for primer selection is the MacVector program from Kodak. In addition to commercially available programs for
15 primer selection, one of skill can easily design simple programs for any of the preferred selection steps. Amplification primers can be selected to provide amplification products that span specific deletions, truncations, and insertions in an amplification target, thereby facilitating the detection of specific abnormalities such as a transposon insertion as described herein.

Antibodies to Afc1p and to Rce1p

Antibodies are raised to Afc1p and Rce1p polypeptides of the present invention, including individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms.
25 Additionally, antibodies are raised to these polypeptides in either their native configurations or in non-native configurations. Anti-idiotypic antibodies can also be generated. Many methods of making antibodies are known to persons of skill. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods
30 are known.

A number of immunogens are optionally used to produce antibodies specifically reactive with Afc1p and Rc1p polypeptides. Recombinant or synthetic polypeptides of 10 amino acids in length, or greater, typically 20 amino acids in length, or greater, more typically 30 amino acids in length, or greater, selected from amino acid sub-sequences of Afc1p and Rc1p are the preferred polypeptide immunogen for the production of monoclonal or polyclonal antibodies. In one class of preferred embodiments, an immunogenic peptide conjugate is also included as an immunogen. Naturally occurring polypeptides are also used either in pure or partially pure form.

Recombinant polypeptides are expressed in eukaryotic or prokaryotic cells and purified using standard techniques. The polypeptide, or a synthetic version thereof, is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies can be generated for subsequent use in immunoassays to measure the presence and quantity of the polypeptide.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (*e.g.*, GST, keyhole limpet hemocyanin, *etc.*), or a polypeptide incorporated into an immunization vector, such as a recombinant vaccinia virus (*see*, U.S. Patent No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired (*see, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY).

Antibodies, including binding fragments and single chain recombinant versions thereof, against whole or predetermined fragments of Afc1p or Rc1p are raised by immunizing animals, *e.g.*, with conjugates of the fragments with carrier proteins as described above. Typically, the immunogen of interest is a peptide of at least about 10 amino acids, more typically the peptide is 20 amino acids in length,

generally the fragment is 25 amino acids in length and often the fragment is 30 amino acids in length or greater. The peptides are optionally coupled to a carrier protein (*e.g.*, as a fusion protein), or are recombinantly expressed in an immunization vector.

Antigenic determinants on selected peptides to which antibodies bind are typically 3 to 10 amino acids in length.

Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies are screened for binding to normal or modified polypeptides, or screened for agonistic or antagonistic activity, *e.g.*, activity mediated through a selected polypeptide. Specific monoclonal and polyclonal antibodies will usually bind with a K_D of at least about .1 mM, more usually at least about 50 μ M, and preferably at least about 1 μ M or better.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, *etc.* Description of techniques for preparing such monoclonal antibodies are found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497. Summarized briefly, this method proceeds by injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secretes a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, retroviruses, or other methods known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection

into the peritoneal cavity of a vertebrate (preferably mammalian) host. The polypeptides and antibodies of the present invention are used with or without modification, and include chimeric antibodies such as humanized murine antibodies.

5 Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors (*see, e.g., Huse et al. (1989) Science 246: 1275-1281; and Ward, et al. (1989) Nature 341: 544-546; and Vaughan et al. (1996) Nature Biotechnology, 14: 309-314*).

10 Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345;
15 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.* (1989) *Proc. Nat'l Acad. Sci. USA* 86: 10029-10033.

20 The antibodies of this invention are also used for affinity chromatography in isolating natural or recombinant Afc1p or Rcelp polypeptides. Columns are prepared, *e.g.,* with the antibodies linked to a solid support, *e.g.,* particles, such as agarose, Sephadex, or the like, where a cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified polypeptides are released.

25 In one highly preferred embodiment, the antibodies are used to screen expression libraries for particular expression products such as homologous proteins to the yeast Afc1p or Rcelp proteins, *e.g.,* in an expression library from human or other mammalian tissue. Optionally, the antibodies in such a procedure are labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

30 Antibodies raised against polypeptides can also be used to raise anti-idiotypic antibodies. These are useful for detecting abnormal growth related to the presence of the respective polypeptides.

Antibodies are optionally humanized. Humanized (chimeric) antibodies are immunoglobulin molecules comprising a human and non-human portion. The antigen combining region (or variable region) of a humanized chimeric antibody is derived from a non-human source (*e.g.*, murine) and the constant region of the chimeric antibody (which confers biological effector function, such as cytotoxicity, to the immunoglobulin) is derived from a human source. The humanized chimeric antibody has the antigen binding specificity of the non-human antibody molecule and the effector function conferred by the human antibody molecule. A large number of methods of generating chimeric antibodies are well known to those of skill in the art (*see, e.g.*, U.S. Patent Nos: 5,502,167, 5,500,362, 5,491,088, 5,482,856, 5,472,693, 5,354,847, 5,292,867, 5,231,026, 5,204,244, 5,202,238, 5,169,939, 5,081,235, 5,075,431, and 4,975,369).

In another embodiment, this invention provides for fully human antibodies against Afc1p or Rce1p polypeptides. Human antibodies consist entirely of characteristically human immunoglobulin sequences. The human antibodies of this invention can be produced in using a wide variety of methods (*see, e.g.*, Larrick *et al.*, U.S. Pat. No. 5,001,065, for a review). A general approach for producing human antibodies by trioma technology is described by Ostberg *et al.* (1983), *Hybridoma* 2: 361-367, Ostberg, U.S. Pat. No. 4,634,664, and Engelman *et al.*, U.S. Pat. No. 4,634,666. Other approaches include immunization of mice transformed to express human immunoglobulin genes, and phage display screening (Vaughan *et al. supra.*).

Afc1p and Rce1p polypeptide assays.

The expression of selected polypeptides (*e.g.*, Afc1p, Rce1p and conservative modifications thereof) can also be detected and/or quantified by detecting or quantifying the expressed polypeptide. As described herein, detection of the various polypeptides of the invention is a feature of certain assays of the invention.

The polypeptides can be detected and quantified by any of a number of means well known to those of skill in the art. These include analytic biochemical methods, such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion

chromatography, and the like, or various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay(RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, western blotting, and the like.

5 In a particularly preferred embodiment, the polypeptides are detected in an electrophoretic protein separation, more preferably in a two-dimensional electrophoresis, while in a most preferred embodiment, the polypeptides are detected using an immunoassay.

10 As used herein, an immunoassay is an assay that utilizes an antibody to specifically bind to the analyte (*e.g.*, selected polypeptide, such as Afc1p or Rc1p). The immunoassay is thus characterized by detection of specific binding of a polypeptide to an anti-polypeptide antibody, as opposed to the use of other physical or chemical properties to isolate, target, and quantify the analyte.

15 As indicated above, the presence or absence of polypeptides in a biological sample can be determined using electrophoretic methods. Means of detecting proteins using electrophoretic techniques are well known to those of skill in the art (*see generally*, R. Scopes (1982) *Protein Purification*, Springer-Verlag, N.Y.; Deutscher, (1990) *Methods in Enzymology Vol. 182: Guide to Protein Purification.*, Academic Press, Inc., N.Y.).

20 In a preferred embodiment, the polypeptides are detected and/or quantified using any of a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology Volume 37: Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); *Basic and Clinical*
25 *Immunology* 7th Edition, Stites & Terr, eds. (1991). Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte. The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds polypeptide(s) or polypeptide subsequences (*e.g.*, antigenic domains which
30 specifically bind to the antibody). In a second preferred embodiment, the capture agent

is the polypeptide and the analyte is antisera comprising an antibody which specifically binds to the polypeptide.

Immunoassays often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled polypeptide or a labeled anti-polypeptide antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/polypeptide complex.

In a preferred embodiment, the labeling agent is a second antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second antibody can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

Other proteins capable of specifically binding immunoglobulin constant regions, such as streptococcal protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see, generally Kronval, et al. (1973) J. Immunol., 111: 1401-1406, and Akerstrom, et al. (1985) J. Immunol., 135: 2589-2542).*

Throughout the assays, incubation and/or washing steps are optionally performed after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

Immunoassays for detecting polypeptides may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte is directly measured. In one preferred "sandwich" assay, for example, the capture agent can be bound directly to a solid substrate where they are immobilized.

The immobilized capture agent then captures analyte present in the test sample. The analyte thus immobilized is then bound by a labeling agent, such as a second antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second antibody can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

In competitive assays, the initial amount of analyte present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte displaced (or competed away) from a capture agent by the analyte present in the sample. In one competitive assay, a known amount of, in this case, analyte is added to the sample and the sample is then contacted with a capture agent. The amount of exogenous analyte bound to the capture agent is inversely proportional to the initial analyte present in the sample.

In a preferred embodiment, western blot (immunoblot) analysis is used to detect and quantify the presence of selected polypeptides in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter) and incubating the sample with the antibodies that specifically bind the selected polypeptide. The antibodies specifically bind to polypeptide on the solid support. These antibodies are optionally directly labeled or alternatively are optionally subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-mouse antibodies) that specifically bind to the selected polypeptide.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (*see*, Monroe *et al.* (1986) *Amer. Clin. Prod. Rev.* 5:34-41). Enzyme linked assays (*e.g.*, ELISA assays) are also preferred.

The assays of this invention as scored (as positive or negative for a selected polypeptide) according to standard methods well known to those of skill in the

art. The particular method of scoring will depend on the assay format and choice of label. For example, a western blot assay can be scored by visualizing the colored product produced by the enzymatic label. A clearly visible colored band or spot at the correct molecular weight is scored as a positive result, while the absence of a clearly visible spot or band is scored as a negative. In a preferred embodiment, a positive test will show a signal intensity (*e.g.*, polypeptide quantity) at least twice that of the background and/or control and more preferably at least 3 times or even at least 5 times greater than the background and/or negative control.

One of skill in the art will appreciate that it is often desirable to reduce non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin.

The particular label or detectable group used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ³H, ¹²⁵I, ³⁵S, ¹⁴C, or ³²P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required,

ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labeling or signal producing systems which may be used, *see*, U.S. Patent No. 4,391,904).

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

As mentioned above, depending upon the assay, various components, including the antigen, target antibody, or anti-antibody, may be bound to a solid surface. Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.* glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass or plastic bead. The desired component may be covalently bound or noncovalently attached through nonspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, are included substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See, for example, *Immobilized Enzymes*, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas (1970) *J. Biol. Chem.* 245 3059).

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labeled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082.

Screening for Nucleic Acids and the Use of Nucleic Acids as Molecular Probes

The nucleic acids of the invention (*e.g.*, *AFCI*, *RCEI*, homologues thereof, and conservative modifications thereof) are useful as molecular probes, in addition to their utility in encoding the polypeptides described herein. As set forth *supra*, certain assays of the invention include the detection of *AFCI* or *RCEI* expression.

Typically, probes derived from the exemplar nucleic acids are used to detect and/or quantitate the presence of complementary nucleic acid in a selected biological sample. A wide variety of formats and labels are available and appropriate for nucleic acid hybridization, including those reviewed in Tijssen (1993) *Laboratory Techniques in biochemistry and molecular biology--hybridization with nucleic acid probes* parts I and II, Elsevier, New York and Choo (ed) (1994) *Methods In Molecular Biology Volume 33- In Situ Hybridization Protocols* Humana Press Inc., New Jersey (*see*

also, other books in the Methods in Molecular Biology series); *see especially*, Chapter 21 of Choo (*id*) "Detection of Virus Nucleic Acids by Radioactive and Nonisotopic *in Situ* Hybridization".

5 For instance, PCR is routinely used to detect nucleic acids in biological samples (*see*, Innis, *supra*, for a general description of PCR techniques). Accordingly, in one class of embodiments, the nucleic acids of the invention are used as PCR primers or templates, or as positive controls in PCR reactions for the detection of in a biological sample. Briefly, nucleic acids with sequence identity or complementarity to an exemplar sequence is used as templates to synthetically produce oligonucleotides of
10 about 15-25 nucleotides with sequences similar or identical to the complement of a the selected nucleic acid subsequence. The oligonucleotides are then used as primers in PCR reactions to detect selected nucleic acids in biological samples. A nucleic acid of the invention (*i.e.*, a cloned nucleic acid corresponding to the region to be amplified) is also optionally used as an amplification template in a separate reactions as a positive
15 control to determine that the PCR reagents and hybridization conditions are appropriate.

Other methods for the detection of nucleic acids in biological samples using nucleic acids of the invention include Southern blots, northern blots, *in situ* hybridization (including Fluorescent *in situ* hybridization (FISH), and a variety of other techniques overviewed in Choo (*supra*)). A variety of automated solid-phase detection
20 techniques are also appropriate. For instance, very large scale immobilized polymer arrays (VLSIPS™) are used for the detection of nucleic acids. *See*, Tijssen (*supra*), Fodor *et al.* (1991) *Science*, 251: 767- 777; Sheldon *et al.* (1993) *Clinical Chemistry* 39(4): 718-719 and Kozal *et al.* (1996) *Nature Medicine* 2(7): 753-759.

25 Therapeutic Uses for Afc1p and Rce1p Inhibitors

In one aspect, the present invention provides therapeutics for treating cancers such as tumors which are correlated to Ras activation. In the therapeutic methods of the invention, a small molecule inhibitor of a mammalian Afc1p or Rce1p protein, such as NMe181, is administered to a patient suffering from cancer and, in
30 particular, cancer associated with an activated Ras oncogene.

Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. Administration is made in any suitable manner, preferably with pharmaceutically acceptable excipients. Suitable methods of administering inhibitors in the context of the present invention to a patient are available. Intra-muscular, subcutaneous and parenteral administration such as intravenous administration are suitable methods of administration. Where the inhibitor is administered to inhibit growth of a tumor, the inhibitor is often administered to the site of the tumor, rather than by systemic introduction. However, systemic introduction is optionally used. Formulations of compositions to be administered can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

Pharmaceutically acceptable excipients are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention. Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time, such as a reduction in the rate of tumor growth, or more preferably, a reduction in tumor size. The dose will be determined by the efficacy of the particular inhibitor employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular inhibitor.

In determining the effective amount of the inhibitor, the physician evaluates circulating plasma levels of the inhibitor, toxicities, progression of the disease, and the production of antibodies to the particular inhibitor.

5 For administration, inhibitors can be administered at a rate determined by the LD-50 of the inhibitor and the side-effects of the inhibitor at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

10 If a patient undergoing infusion of an inhibitor develops fevers, chills, or muscle aches, he/she typically receives the appropriate dose of aspirin, ibuprofen or acetaminophen. Patients who experience reactions to the infusion such as fever, muscle aches, and chills are premedicated 30 minutes prior to the future infusions with either aspirin, acetaminophen, or diphenhydramine. Meperidine is used for more severe chills and muscle aches that do not quickly respond to antipyretics and antihistamines. Infusion is slowed or discontinued depending upon the severity of the reaction.

15 The effect of the therapeutic inhibitors are measured by monitoring the size of a tumor or the extent of metastasis before treatment, and comparing the size of the tumor or extent of metastasis in a patient over time. Typically, measurements are taken before, during and after the therapeutic regimen.

20 Kits

The present invention provides a variety of kits for the detection of *AFC1* or *RCE1* nucleic acids or proteins, and for the testing compounds for their ability to inhibit *AFC1* or *RCE1* expression, or Afc1p or Rce1p protein activity.

25 Detection kits preferably include one or more reagents for determining the presence or absence of a selected nucleic acid or protein, *i.e.*, any of the nucleic acids or proteins described herein. Preferred reagents include nucleic acid probes that specifically hybridize to the exemplar sequences, or subsequence thereof; probes that specifically bind to an abnormal genes (*e.g.*, one containing premature truncations, insertions, or deletions), and antibodies that specifically bind to polypeptides or
30 subsequences thereof. The antibody or hybridization probe may be free or immobilized on a solid support such as a test tube, a microtiter plate, a dipstick and the like. The kit

optionally include instructional materials teaching the use of the antibody or hybridization probe in an assay for the detection of the relevant nucleic acid or protein, a container or other packaging material or the like.

5 The kits optionally include alternatively, or in combination with any of the other components described herein, an antibody which specifically binds a polypeptide of the invention. The antibody can be monoclonal or polyclonal. The antibody can be conjugated to another moiety such as a label and/or it can be immobilized on a solid support (substrate).

10 The kits can also optionally include a second antibody for detection of polypeptide/antibody complexes or for detection of hybridized nucleic acid probes. The kits optionally include appropriate reagents for detection of labels, positive and negative controls, washing solutions, dilution buffers and the like.

15 Kits testing for inhibition of *RCE1* or *AFC1* expression optionally include any of the components described above for detecting nucleic acids or proteins. Kits testing for *Afc1p* or *Rce1p* activity can monitor the proteolytic cleavage of the terminal AAX peptide from a relevant CAAX protein such as Ras. This can be accomplished by monitoring the change in electrophoretic mobility, *e.g.*, in a western blot or ELISA assay. These kits optionally include any of the following: reagents for detecting a CAAX protein (*e.g.*, anti-Ras antibodies), electrophoretic equipment, instructions in the
20 detection of AAX cleavage or the like. Other assays for monitoring protease activity are described in Ashby and Rine "Ras and a- Factor Converting Enzyme" (1995) *Methods in Enzymology* 230:235.

25 In addition to monitoring AAX cleavage directly, the present invention provides for the detection of AAX cleavage using a functional assay. For example, as described herein, a heat-shock assay can be used to monitor prenylation-dependent Ras activity. Kits optionally include any of the components used in such an assay, including yeast strains such as $\Delta AFC1$ or $\Delta RCE1$, instructions, containers, growth media, control yeast strains, thermocycling equipment, water baths for administering the heat shock, and the like.

30

Discussion of the Accompanying Sequence Listing

SEQ ID NO:1 provides the sequence of the *AFCI* gene from yeast. SEQ ID NO:2 provides the sequence of the *RCEI* gene from yeast. In each case, the information is presented as a DNA sequence. One of skill will readily understand that the sequence also describes the corresponding RNA (*i.e.*, by substitution of the T residues with U residues) and a variety of conservatively modified variations thereof. In addition, the nucleic acid sequence provides the corresponding amino acid sequence by translating the given DNA sequence using the genetic code.

SEQ ID NO:3 provides the protein sequence of the Afc1p protein from yeast. SEQ ID NO:4 provides the sequence of the Rce1p protein from yeast. In each case, the information is presented as a polypeptide sequence. One of skill will readily understand that the sequences also describe all of the corresponding RNA and DNA sequences which encode the protein, by conversion of the amino acid sequence into the corresponding nucleotide sequence using the genetic code, by alternately assigning each possible codon in each possible codon position. The sequences also provides a variety of conservatively modified variations by substituting appropriate residues with the exemplar conservative amino acid substitutions provided, *e.g.*, in the Definitions section above.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially similar results.

To evaluate the functional role of Ras CAAX proteolysis, naturally occurring CAAX sequences from rabbit skeletal muscle phosphorylase kinase were utilized. The α and β subunits of this holoenzyme contain CAAX sequences that *in vivo* are prenylated, yet do not undergo further processing (Heilmeyer, *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**(20):9554-9558 (1992)). It has now been demonstrated that peptides containing these sequences are not proteolytically cleaved *in vitro* by the membrane-bound CAAX protease. These same peptides do, however, bind to the protease as

defined by their ability to compete for proteolysis of peptides with normally processed CAAX sequences.

The issue regarding whether CAAX proteolysis and methylation are required for yeast Ras2p function has been addressed. To do so, a highly sensitive and reproducible assay of Ras2p function has been developed. One of the hallmark phenotypes of activating mutations in yeast Ras2p is the inability of the cells to properly enter stationary phase resulting in poor viability and heat shock sensitivity. Current heat shock assays to assess Ras activation utilize plate assays where a large number of cells being tested are transferred to plates that have been prewarmed to 55°C. The plates are then incubated at 55°C for various times up to one hour. After a subsequent incubation at 30°C for about 18 hours, the strains are scored for yeast death or growth and, thus, heat shock sensitivity or resistance, respectively. This assay is adequate for crude measurements of heat shock sensitivity, but, unfortunately, it is clearly inadequate for quantitating intermediate degrees of heat shock sensitivity.

As such, an improved assay of Ras2p function has been developed. This assay utilizes liquid-based heat shock of the yeast strains in, for example, a programmable thermocycle (PCR) machine or other water-bath. Generally, cells are grown in a liquid medium into a stationary phase. The liquid medium can be water or some other growth medium, such as the commercially available Yeast Minimal Medium (See, e.g., Atlas, *et al.*, *HANDBOOK OF MICROBIOLOGICAL MEDIA* (CRC Press, Ann Arbor, Michigan (1993); *see also*, Sambrook, Ausbel and Berger, *all supra*). Equal amounts of cells are added to tubes containing the liquid medium, preferably water. At the same time, portions of these suspensions are plated on the growth media to score the viability before the heat shock, *i.e.*, to serve as controls. The tubes are then placed in a PCR machine or a water-bath at a temperature ranging between about 40°C and about 60°C, more preferably at a temperature between 50°C and 55°C, and heat shocked for a time period ranging from about 0.5 minute to about 20 minutes, more preferably for a time period from about 3 minutes to about 12 minutes. It will be readily apparent to those of skill in the art that the specific reaction conditions employed will depend upon the amount of cells in the suspension and upon how deep into the stationary phase the tested cells are.

The heat-treated cells are then cooled down to a temperature ranging from 0°C to about 30°C, more preferably to about room temperature (20°C), and plated on the growth media to score viability after the heat shock. The ratio of the number of survivors in the tested strain (*e.g.*, a strain carrying deletions in *AFC1* or *RCE1* or alterations in the *RAS2* C-terminus CAMQ sequence) to the number of survivors in the control strain (*i.e.*, the strain which has no deletions or alterations) defines the level of suppression of heat shock sensitivity. At the same time, the strains with plasmids carrying the wild type *RAS2* gene are also tested for heat shock sensitivity to determine if the introduced changes by themselves have any effect on the rate of survival.

To determine the functional impact of blocking Ras2p CAAX proteolysis, the normal Ras2p CAAX sequence CIIS was replaced with CAMQ or CLVS using site-directed mutagenesis. These non-proteolyzable sequences reduced the activated phenotype of cells approximately 100-fold. In contrast, the presence of these non-proteolyzable CAAX sequences did not have any detectable phenotype in non-activated forms of Ras2p. These results are the first demonstration of a functional requirement of Ras-CAAX proteolysis and indicate that this aspect of Ras processing represents a pharmacological target in tumors containing activating mutations in Ras.

In addition to testing the effects of the non-proteolyzable rabbit phosphorylase kinase CAAX sequences CAMQ and CLVS on Ras2p function, the importance of Ras2p C-terminal methylation was also tested. The *STE14* gene product has been shown to encode the prenylated protein C-terminal methyltransferase that processes both the Ras2p protein and the pheromone α -factor (Hrycyna, *et al.*, *EMBO J.*, 10:1699-1709 (1991)). Deletion of the *STE14* structural gene results in an absence of detectable Ras2p methylation, but does not effect activated Ras2p heat shock sensitivity when evaluated with the standard plate heat shock assay (*Id.*).

The importance of C-terminal methylation of activated Ras2^{val19} was assessed in a strain in which the *STE14* gene was replaced with the *LEU2* gene (*ste14 Δ ::LEU2*) in the liquid-based heat shock assay described above. As was the case with blocking CAAX proteolysis with the CAMQ or CLVS substitution, preventing C-terminal methylation reduced heat shock sensitivity greater than 50-fold. This result indicated that blocking activated Ras2p methylation significantly reduced Ras2p function

and, thus, attenuated the activated phenotype. This result is the first demonstration of the functional relevance of Ras2p farnesylated cysteine methylation.

In addition to the foregoing, the issue of whether the effects of blocking Ras2p proteolysis and blocking Ras2p methylation would be additive when combined was tested. For this experiment, heat shock sensitivities in strains with wild-type or activated forms of Ras2p with various CAAX sequences in the presence or absence of the *STE14* gene were compared. The results indicate that the heat shock resistance conferred by the non-proteolyzable CAAX sequence together with the absence of the *STE14* gene (*ste14Δ::LEU2*) does not result in an increased heat shock resistance beyond that of each component alone. These data therefore support the notion that CAMQ and CLVS represent non-proteolyzable sequences and that the two phenotypes, namely heat shock resistance in Ras2^{val19} CAMQ or CLVS and heat shock resistance in the Ras2^{val19} (*ste14Δ::LEU2*) strains, are epistatic.

As a means to isolate mutants in CAAX processing, a powerful genetic selection for mutants defective in a-factor processing was performed. The a-factor pheromone is another CAAX containing peptide that is processed by the same protein:farnesyltransferase and methyltransferase enzymes as Ras2p. The genetic selection produced a novel mutant that displayed a differential phenotype depending upon the particular AAX extension of the CAAX sequence. This mutant possessed a single mutation that was responsible for the phenotype and the corresponding gene was cloned by complementation. The gene, named *AFC1* for a-Factor Convertase, contains an amino acid sequence motif identified as the active site in several characterized zinc metalloproteases. This property is consistent with both the *in vitro* proteolysis defect of the corresponding mutant and the *o*-phenanthroline sensitivity of this activity in wild-type extracts. Furthermore, this a-factor CAAX protease was dependent upon a farnesylated substrate as expected of a protease that processes a-factor *in vivo*. The isolation of *AFC1* represents the first CAAX protease to be identified. A genetic knockout of *AFC1* resulted in a significant, but not complete, reduction of a-factor production, thereby exposing the presence of a second CAAX protease. Using a similar approach, a second CAAX protease was identified. This gene for the second protease has been named *RCE1* (Ras and a-Factor Converting Enzyme).

Example 1: Autocrine Arrest Selection

Autocrine arrest selection was designed to simplify the task of identifying mutants that are defective in the production of yeast mating pheromone- a-factor. This assay relies on the fact that haploid yeast cells of both a and α mating types share the same mating signal transduction pathway. The mating specificity is defined only by the type of mating pheromone receptor that is expressed on the surface of the cell. Expression of the a-factor receptor in a cell producing biologically active a-factor will lead to autocrine arrest. Mutants having mutations in the genes responsible for either a-factor production or for transduction of the mating signal will be able to escape the arrest, grow and form colonies.

A. Strain Design

The SST2 gene (mutations in this gene confer supersensitivity to the mating pheromone, thereby allowing tighter arrest) was deleted using a two-step gene replacement in the JRY3658 strain (W303 *HML Δ p*, *HMR Δ p*, *MAT Δ p*). A plasmid carrying the *MAT α* locus was introduced in the JRY3658 Δ sst2 strain allowing it to mate as an α strain. JRY3658 Δ sst2 *pMAT α* was crossed to JRY527 Δ *mfa1::hisG*, *mfa2 Δ ::hisG*. The diploid was sporulated and the segregant of the following genotype was identified: *HML α Δ p*, *HMR α Δ p*, *MAT α Δ p*, Δ *mfa1::hisG*, *mfa2 Δ ::hisG*, Δ *sst2*, *his3*, *lys2*, *leu2-3,112*, *ura3*.

The gene encoding the a-factor receptor (*i.e.*, STE3) was placed under the control of the inducible GAL promoter, thereby allowing the expression of the gene to be controlled by the type of carbon source in the growth medium. This construct was integrated in the STE3 locus of the strain described above. A plasmid carrying MFA1 gene with the altered C-terminal CAAX sequence, *i.e.*, CAMQ, was used as a source of the mating pheromone. This sequence was introduced by site directed mutagenesis.

B. Selection

In each round of selection, 20 independent colonies were picked and grown at 30°C in the supplemented minimal media. Equal numbers of cells from each culture were mutagenized by UV light in liquid. The mutagenized cells were

transferred back into the growth media and grown overnight at room temperature to overcome the phenotypic lag. Cells were plated on plates containing galactose to induce expression of the a-factor receptor construct, and incubated at room temperature.

5 C. *Identification of the Mutants*

10 All colonies formed on galactose-containing media were scored for a-factor production in halo assays. Mutant strains which produced no or very little of the a-factor were used in a secondary screen. In the secondary screen, patches of cells were treated with synthetic alpha factor to eliminate all the mutants in the mating signal transduction pathway. Strains which were able to arrest (indicating that they were able to grow on galactose not because they couldn't respond to the pheromone) were used in complementation tests. The mutants were crossed to the strains with defects in the genes known to be involved in production of a-factor (*i.e.*, *ram1* - a subunit of the farnesyltransferase, *ste14* - methyltransferase and *ste6* - a-factor transporter). The strains 15 which were complemented by all the mutant strains were used in another complementation test, using plasmids carrying the a-factor processing genes (*i.e.*, *RAM1*, *RAM2*, *STE14* and *STE6*).

20 The mutant strains which passed both complementation tests were analyzed for substrate specific processing defects. The plasmid with the *MFA1(CAMQ)* variant, used in the selection, was cured from the strains and a wild type *MFA1(CVIA)* gene carrying plasmid was introduced. The mutant strains which produced no a-factor with *MFA1(CAMQ)* plasmid and which were able to produce detectable amounts of biologically active a-factor with *MFA1 (CVIA)* formed a large complementation group termed *AFC1* (a-Factor Convertase). The gene defined by this complementation group 25 was cloned using a multicopy genomic library by complementation of the mutant phenotype (a-factor production). The *RCE1* (Ras and a-Factor Converting Enzyme) was identified as a partial multicopy suppressor of the *afc1 MFA1(CAMQ)* dependent mutant phenotype.

Example 2: Heat Shock Sensitivity Assay

The heat shock sensitivity assay is used to determine if changes introduced in the yeast strain have any effects on Ras signaling. This assay utilizes a dominant, hyperactivated allele of *RAS2*, *i.e.*, *RAS2^{val19}*. Cells carrying the *RAS2^{val19}* allele on a CEN plasmid are significantly more sensitive to the short term heat treatment than wild type strains. The assay can be performed in many different ways. The goal of the assay is to evaluate the number of cells able to survive the heat shock.

The original assay involves streaking similar numbers of cells on a plate which is then placed in a water-bath at a temperature of about 50° to 55°C for a time period of about 10 to 30 min. Unfortunately, this assay can reveal only major differences in heat shock sensitivity. It has, however, been discovered that modifications introduced to this assay allow more precise quantitative evaluation of the differences in the survival rate. In the modified assay, cells are grown in a liquid medium into a stationary phase. The liquid medium can be water or some other growth medium, such as the commercially available Yeast Minimal Medium (*See, e.g.*, Atlas, *et al.*, *HANDBOOK OF MICROBIOLOGICAL MEDIA* (CRC Press, Ann Arbor, Michigan (1993))). Equal amounts of cells are added to tubes containing the liquid medium, preferably water. At the same time, portions of these suspensions are plated on the growth media to score the viability before the heat shock, *i.e.*, to serve as controls. The tubes are then placed in a PCR machine or a water-bath at a temperature ranging between about 40°C and about 60°C, more preferably at a temperature between 50°C and 55°C, and heat shocked for a time period ranging from about 0.5 minute to about 20 minutes, more preferably for a time period from about 3 minutes to about 12 minutes. It will be readily apparent to those of skill in the art that the specific reaction conditions employed will depend upon the amount of cells in the suspension and upon how deep into the stationary phase the tested cells are.

The heat-treated cells are then cooled down to a temperature ranging from 0°C to about 30°C, more preferably to about room temperature (20°C), and plated on the growth media to score viability after the heat shock. The ratio of the number of survivors in the tested strain (*e.g.*, a strain carrying deletions in *AFC1* or *RCE1* or alterations in the *RAS2* C-terminus CAMQ sequence) to the number of survivors in the

control strain (*i.e.*, the strain which has no deletions or alterations) defines the level of suppression of heat shock sensitivity. At the same time, the strains with plasmids carrying the wild type *RAS2* gene are also tested for heat shock sensitivity to determine if the introduced changes by themselves have any effect on the rate of survival.

5 In a variation of the above assay, all tested strains are transferred to a 96-well microtiter plate and serially diluted 5-fold 5 times. An aliquot (about 6 μ l) from each well is placed on the growth media to determine the viability of the strain. The plate is placed in a water-bath at a temperature of about 50°C to about 55°C. Aliquots are removed after 7 and 12 minutes of heat treatment. The heat shock sensitivity is
10 scored by determining which of the lowest dilutions still has survivors.

An additional assay to measure *RCE1* function in Ras processing was to test growth at semi-permissive conditions of cells contains the Ras temperature sensitive mutant. *RCE1* mutants decreased the maximum permissive temperature for growth. In one of the experiments carried out, 2 ODs ($\sim 2 \times 10^7$ cells) of cells without the *RAS2^{val19}* plasmid and 3.5 ODs of cells with the *RAS2^{val19}* plasmid were placed in the first row of
15 the plate. Each row thereafter represents a 5-fold dilution. Cells were incubated at a temperature of about 50°C for about 7 minutes. Longer incubation times showed even more dramatic effects. Heat shock sensitivity was reduced 5-fold. The following assay was used to measure suppression. Strains were grown in YM + supplements for about
20 2.5 days to an OD of about 2.6×10^6 . Cells from each strain (in duplicate) carrying the *RAS2^{val19}* plasmid were resuspended in 1 ml of water. 50 μ l of each of the suspension was plated for viability. The tubes were then incubated for about 10 min at a temperature of about 50°C. After being cooled down to about room temperature, 650 μ l and 150 μ l aliquots were plated on the growth media to score the rate of survival.

Example 3: Yeasts Strains Used In The Various Assays

A. *Strains Used In Temperature Sensitivity Experiments*

The strains used in the temperature sensitivity experiments were obtained
30 by crossing the W303 MAT α Δ *afc1*, Δ *rce1* strain to W303 MAT α *ras1::HIS3*, *ras2-23^{ts}* (PHY 1150).

Analysis of the segregants from the cross yielded the following strains:

Wild type: W303, *MATa*, *ras1::HIS3*, *ras2-23^u*, *ade2*, *leu2*, *his3*,
trp1, *ura3*
 Δ *afcl*: W303, *MATa*, *ras1::HIS3*, *ras2-23^u*, *ade2*, Δ *afcl::HIS3*,
leu2, *his3*, *trp1*, *ura3*
 Δ *rce1*: W303, *MATa*, *ras1::HIS3*, *ras2-23^u*, *ade2*, Δ *rce1::TRP1*,
leu2, *his3*, *trp1*, *ura3*
 Δ *afcl*, Δ *rce1*: W303, *MATa*, *ras1::HIS3*, *ras2-23^u*, *ade2*, Δ *afcl::HIS3*,
 Δ *rce1::TRP1*, *leu2*, *his3*, *trp1*, *ura3*

B. Strains Used In Heat Shock Assays:

All strains were derived from the common laboratory strain W303 (JRY2334). *AFC1* was deleted by homologous recombination using the construct in which a part of the open reading frame (ORF) is replaced with the *HIS3* gene. For a discussion regarding the use of homologous recombination, see, e.g., Scherer, S., et al., "Replacement of chromosome segments with altered DNA sequences constructed in vitro," *Proc. Natl. Acad. Sci. USA*, 76(10):4951-5 (1979). *RCE1* was deleted using homologous recombination using a PCR product in which the coding region of *TRP1* gene is flanked by ~40 bp sequences homologous to the sequences just upstream and downstream of the ORF encoding the *RCE1* gene.

A double deletion strain was obtained by crossing Δ *afcl* *MATa* strain to Δ *rce1* *MATa* strain, followed by dissection of the sporulated diploid and analysis of the segregants.

Genotypes:

Wild type: W303, *MATa*, *leu2*, *his3*, *trp1*, *ura3*
 Δ *afcl*: W303, *MATa*, Δ *afcl::HIS3*, *leu2*, *his3*, *trp1*, *ura3*
 Δ *rce1*: W303, *MATa*, Δ *rce1::TRP1*, *leu2*, *his3*, *trp1*, *ura3*
 Δ *afcl*, Δ *rce1*: W303, *MATa*, Δ *afcl::HIS3*, Δ *rce1::TRP1*, *leu2*, *his3*, *trp1*,
ura3

The *RAS2^{val19}* allele was introduced in these strains on *URA3* CEN plasmid.

Control strains carried *URA3* CEN plasmid with wild type *RAS2* gene.

- 5 The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Rine, Jasper D.
Boyartchuk, Victor L.
Ashby, Matthew N.
- (ii) TITLE OF INVENTION: AFC1 AND RCE1 ISOPRENYLATED CAAX
PROCESSING ENZYMES
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Pennie & Edmonds LLP
 - (B) STREET: 1155 Avenue of the Americas
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 10036
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Mandel, SaraLynn
 - (B) REGISTRATION NUMBER: 31,853
 - (C) REFERENCE/DOCKET NUMBER: 9067-008-999
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (415) 493-4935
 - (B) TELEFAX: (415) 493-5556
 - (C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1825 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACCTACCTTT TTTTCTATCT TCAACAACGA AACGCCTTAC ACACACACAC ACATACATCT	60
ACATACATAC ATACAAATAT ACATATATGT AAAGTTGTAT ATTCATTCCT ATTAACCAAA	120
AAGAGGCAAT TAAACTTTTC CCTCTTTTTC TACGTCATTT ACTCAAAAAC TCTAATTCCT	180
TCGTCTCTGT TCTGCCATTT TCTCCAGAAA AAAATCGACG GGAAATAAAA AAAAAAGAC	240
AACGAACAAG AGAAAAAGTT CGCGAATTAT AAACCACTTC TATAATTAAC AGGAAAAGGA	300

AGGAAAAAAAA	AGGAGGAAAA	GAAAACTGCA	GGCCTTTATT	CATGTTTGAT	CTTAAGACGA	360
TTCTCGACCA	TCCTAATATC	CCGTGGAAAT	TAATCATTTT	TGGGTTCTCG	ATTGCCCAAT	420
TTTCTTTTCGA	ATCTTACTTG	ACGTACAGAC	AGTACCAGAA	GCTATCTGAA	ACAAAGTTGC	480
CACCTGTGCT	GGAAGACGAA	ATTGATGATG	AACTTTTTC	TAAATCAAGG	AACACTCCC	540
GGGCCAAGGC	CAAGTTCTCC	ATTTTCGGTG	ACGTCTATAA	CCTAGCCCAA	AAGCTAGTTT	600
TCATCAAATA	CGACCTCTTC	CCTAAAATCT	GGCACATGGC	CGTTTCTTTA	TTGAATGCAG	660
TCCTGCCAGT	CAGATTTCAT	ATGGTCTCCA	CTGTCCGACA	GAGTTTTATG	CTTCTTGGGT	720
CTCTTATCCA	GTTTGTCTAC	CTTGGTTGAT	TTGCCACTCT	CTTACTATAG	CCATTTTGTC	780
CTGGAAGAAA	AATTTGGTTT	CAATAAATTG	ACCGTCCAAC	TATGGATCAC	CGATATGATC	840
AAGAGTCTGA	CTTTGGCGTA	TGCTATTGGT	GGCCCAATCC	TTTACCTGTT	CCTTAAGATC	900
TTTGATAAAT	TCCCTACTGA	TTTCCTTTGG	TACATTATGG	TCTTCTTGTT	CGTTGTCCAA	960
ATCTTAGCCA	TGACAATCAT	TCCAGTCTTC	ATCATGCCCA	TGTTTAATAA	GTTCACTCCA	1020
TTGGAGGACG	GTGAACTGAA	AAAATCTATT	GAAAGTTTGG	CCGATAGAGT	TGGGTTCCCT	1080
CTAGATAAGA	TTTTTGTCAT	TGACGGCTCA	AAAAGATCTT	CTCATTCAA	CGCATATTTT	1140
ACAGGTTTGC	CATTCACCTC	CAAGAGAATT	GTTTTGTTCG	ACACTTTAGT	GAACAGTAAT	1200
TCTACTGATG	AAATTACGGC	TGTTTTGGCC	CATGAAATCG	GTCCTGGCA	AAAAAACCAC	1260
ATCGTTAATA	TGGTCATCTT	TAGTCAATTG	CACACCTTCC	TCATTTTCTC	CCTTTTCACC	1320
AGCATCTACA	GAAATACATC	ATTTTACAAC	ACCTTCGGCT	TTTTCTTAGA	GAAGTCCACT	1380
GGCAGTTTTG	TTGATCCCGT	TATCACTAAG	GAATTCCCCA	TTATCATTGG	ATTTATGTTA	1440
TTTAACGACT	TATTAACCTC	ACTCGAATGT	GCCATGCAAT	TCGTGATGAG	TTTAATTTCC	1500
AGAACTCATG	AATATCAAGC	TGATGCTTAT	GCTAAAAAAT	TGGGCTACAA	GCAAAATCTA	1560
TGTAGGGCTC	TAATTGATCT	ACAAATCAAA	AACCTTTCCA	CCATGAATGT	AGATCCTCTG	1620
TATTCTAGCT	ATCATTATTC	CCATCCAAC	CTAGCTGAAA	GATCGACCGC	TCTAGACTAT	1680
GTTAGTGAAA	AGAAGAAAAA	CTAATCTATA	GAGTACACAT	ATTAGCATGT	ACCGTTAAAT	1740
TCAGCTTCGT	TATGTCTATA	TCTACATACA	TACACAGGTA	TCTACTATAA	GAATAAAGGA	1800
AAGAAAAAAT	AAACGATTAA	ACATT				1825

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2850 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGAACTGTTG	ATGAACAAAG	AGAAGCTGAC	AAGCATCAAA	GCTTTGTACG	ATGATTTC	60
------------	------------	------------	------------	------------	----------	----

TTCAAAAATT	TGTGAATATG	AAACCAAGTT	CAACAAGAAT	TTTCTTGAAT	TAAATGAGTT	120
ATATAATATG	AATAGGGGAG	ACCGTAGGCC	AAAGGAACTG	AAATTTACAG	ATTTTATTAC	180
TTCACAGCTG	TTTAACGATA	TCGAAAGCAT	TTGCAACTTG	AAAGTTAGTG	TTCACTAATT	240
ATCCAACATT	TTTAAAAAAC	AGGTCAGTAC	CCTAAAACAA	CACTCAAAGC	ACGCATTATC	300
TGAGGATTCA	ATATCGCACA	CAGGTAACGG	TAGTTCATCG	TCGCCCAGTT	CAGCGTCATT	360
AACGCCAGTA	ACTTCTTCAT	CCAAGAGTAG	TTTATTTTTTA	CCTAGCGGTA	GCTCGTCTAC	420
TTCCCTGAAA	TTTACAGACC	AGATTGTTCA	TAAATGGGTT	AGGATTGCTC	CTTTACAGTA	480
CAAACGAGAC	ATTAATGTGA	ACTTGGAATT	TAATAAGGAC	ATTAAGGAAA	CTTTAATTCC	540
AAGTTTTGAA	AGCTGCCTAT	GTTGTAGGTT	TTATTGCGTT	CGAGTAATGA	TTAAATTTGA	600
AAACCATCTT	GGCGTAGCGA	AGATTGATAT	CCCTATTTCT	GTTAGGCAAG	TGACAAAATA	660
AAAAAACATT	AGAAAAAATT	CTCGTTACTT	TTCTTATAGA	TATAGATATA	TGTATGGTTT	720
GCTTATAGAT	GAAGGTATTT	ATCGCGTCCT	TTGTATTCCC	TATTATTAAT	AAAATTCTTT	780
TAAATGCAT	TTTCTGGTGC	TCTTTTGTTG	CTTCTGTATT	TTTTTTTTTT	TGGACCACTG	840
GATGGAAAAC	CTTTGATGAT	TTTATTACCT	TTATTTTAAAC	TTACTAAAAT	ATCGAGATTT	900
CAGGAACAAA	ACATAGAATT	TTCTTTGTCA	AGAAAAATAA	AACGAAATAA	ATTGATGCTT	960
TGACTACTGA	CTGTCTGTCA	TAGAGAGAAC	CAGAACAGCA	ATGCTACAAT	TCTCAACATT	1020
TCTAGTGCTC	CTATACATCT	CCATATCCTA	TGTGCTACCG	CTATATGCAA	CTTCACAACC	1080
AGAAGGGTCT	AAACGAGATA	ATCCTCGAAC	GATTAAATCT	CGCATGCAAA	AACCTTACAAT	1140
TATGCTAATT	TCCAACCTTT	TTTTGGTGCC	TTTTTTTACAA	TCTCAATTAT	CTAGTACCAC	1200
TTACATATA	AGTTTCAAGG	ACGCATTTTT	AGGCTTAGGT	ATTATCCCAG	GTTATTACGC	1260
TGCATTGCCA	AACCCTTGGC	AATTCAGCCA	GTTTCGTGAA	GACTTAACGA	AATGTGTTGC	1320
GATGTTATTG	ACCTTATATT	GTGGACCCGT	TTTAGATTTT	GTATTATATC	ATTTATTAAA	1380
TCCAAAGAGC	TCTATACTTG	AAGATTTTTA	CCATGAATTC	CTGAATATTT	GGAGTTTCAG	1440
GAATTTTATA	TTTGACCAA	TAAGTGAAGA	AATATTTTAC	ACGTCAATGC	TTTTGACTAC	1500
GTACTTAAAC	CTAATACCGC	ATTCGCAACT	AAGCTATCAA	CAGTTATTTT	GGCAACCATC	1560
GCTTTTTTTT	GGACTTGCGC	ACGCACACCA	TGCTTATGAG	CAATTACAGG	AAGGCTCCAT	1620
GACAACTGTT	TCCATTCTGC	TGACAACATG	CTTCCAAATT	TTATACACAA	CACTTTTTGG	1680
AGGGTTAACC	AAGTTTGTAT	TGCAATATCA	TGGGGTTTTAA	TGGTCCTTCA	AGATTGAATT	1740
TACATTTTAC	AGTAGTAGAC	AAGAAAGCTG	GACGCATTTT	CAAATTGGTC	TCTCAATCTG	1800
GAATAAGTGC	TACTTCGCAC	TGCTGGTCCT	TGGATTAATA	TCCCTGAAGG	ATACCTTACA	1860
AACTCTGGTA	GGAACCTCTG	GTTATAGAAT	AACCCTTTAG	CCTTTTTTTAC	GTAATTGTAT	1920
ACCGTTTAAA	ATTTCTATAG	TACTATAACC	TTTTTTTCACT	ACTATTATGG	AATTCTATCG	1980
AGCGACCGGG	CTTTTGTTAC	GGAAGAGTGA	AAAAATCGAG	TTTTGGTGTT	TTGGTGAAAG	2040
AATTTGGAGG	ACTATAAAGT	ACCTATACTT	TGTATTACGG	ACTCAATAAC	AAGTCGTTTCG	2100

TGTCAGTGGT	ATTGAAGTTG	TCAGATCTAA	GAGTAGAGAG	AAGGTGGCAT	CTAATAGGTT	2160
TCGACGTTTT	TCTTTTTTTT	AGGTTTTTAT	TTGGTCTCCT	AGAATTTAAG	GTCTTAGTTA	2220
GTTTTGGTTT	GTTTTGTGGG	TTACATATTT	TCAATTCAAA	GGAGAATTTA	GCTGTCTTTT	2280
ATAATGTCCA	ATAGAGATAA	CGAGAGCATG	CTGCGTACTA	CATCAAGCGA	TAAGGCGATC	2340
GCTAGTCAAA	GGGATAAACG	GAAGTCTGAA	GTTTTGATTG	CTGCACAGTC	CCTTGACAAT	2400
GAAATCCGCA	GCGTAAAAAA	CCTAAAAAGA	TTGTTCGATTG	GGTCAATGGA	TTTACTTATT	2460
GATCCAGAAT	TAGATATAAA	ATTCGGTGGG	GAATCTAGTG	GGAGACGATC	ATGGTCTGGC	2520
ACGACATCCA	GTTCTGCGTC	AATGCCAAGT	GACACAACCA	CCGTTAATAA	CACACGATAT	2580
AGCGATCCAA	CTCCGCTAGA	GAAC TTGCAT	GGGAGGGGTA	ACTCAGGGAT	AGAATCCTCC	2640
AATAAGACTA	AAATTAAATG	CTAACGTATT	AAAGAAAAAC	TTATTATGGG	TTCCCGCCAA	2700
TCAACACCCT	AACGTTAAGC	CTGATAATTT	CCTAGAGCTT	GTACAAGATA	CTTTACAAAA	2760
TATACAAC TA	AGCGACAATG	GTGAAGATAA	TGATGGGAAT	AGCAATGAAA	ATAACGATAT	2820
TGAGGATAAT	GGGGAGGATA	AAGAATCACA				2850

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 451 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..451
- (D) OTHER INFORMATION: /note= "The protein sequence of the Afc1p protein from yeast presented as a polypeptide sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Phe	Asp	Leu	Lys	Thr	Ile	Leu	Asp	His	Pro	Asn	Thr	Pro	Trp	Lys
1				5					10					15	
Leu	Ile	Ile	Ser	Gly	Phe	Ser	Ile	Ala	Gln	Phe	Ser	Phe	Glu	Ser	Tyr
			20					25					30		
Leu	Thr	Tyr	Arg	Gln	Tyr	Gln	Lys	Leu	Ser	Glu	Thr	Lys	Leu	Pro	Pro
			35				40					45			
Val	Leu	Glu	Asp	Glu	Ile	Asp	Asp	Glu	Thr	Phe	His	Lys	Ser	Arg	Asn
			50			55					60				
Tyr	Ser	Arg	Ala	Lys	Ala	Lys	Phe	Ser	Ile	Phe	Gly	Asp	Val	Tyr	Asn
			65			70				75				80	
Leu	Ala	Gln	Lys	Leu	Val	Phe	Ile	Lys	Tyr	Asp	Leu	Phe	Pro	Lys	Ile
			85						90					95	

Trp His Met Ala Val Ser Leu Leu Asn Ala Val Leu Pro Val Arg Phe
 100 105 110
 His Met Val Ser Thr Val Ala Gln Ser Leu Cys Phe Leu Gly Leu Leu
 115 120 125
 Ser Ser Leu Ser Thr Leu Val Asp Leu Pro Leu Ser Tyr Tyr Ser His
 130 135 140
 Phe Val Leu Glu Glu Lys Phe Gly Phe Asn Lys Leu Thr Val Gln Leu
 145 150 155 160
 Trp Ile Thr Asp Met Ile Lys Ser Leu Thr Leu Ala Tyr Ala Ile Gly
 165 170 175
 Gly Pro Ile Leu Tyr Leu Phe Leu Lys Ile Phe Asp Lys Phe Pro Thr
 180 185 190
 Asp Phe Leu Trp Tyr Ile Met Val Phe Leu Phe Val Val Gln Ile Leu
 195 200 205
 Ala Met Thr Ile Ile Pro Val Phe Ile Met Pro Met Phe Met Lys Phe
 210 215 220
 Thr Pro Leu Glu Asp Gly Glu Leu Lys Lys Ser Ile Glu Ser Leu Ala
 225 230 235 240
 Asp Arg Val Gly Phe Pro Leu Asp Lys Ile Phe Val Ile Asp Gly Ser
 245 250 255
 Lys Arg Ser Ser His Ser Asn Ala Tyr Phe Thr Gly Leu Pro Phe Thr
 260 265 270
 Ser Lys Arg Ile Val Leu Phe Asp Thr Leu Val Asn Ser Asn Ser Thr
 275 280 285
 Asp Glu Ile Thr Ala Val Leu Ala His Glu Ile Gly His Trp Gln Lys
 290 295 300
 Met His Ile Val Asn Met Val Ile Phe Ser Gln Leu His Thr Phe Leu
 305 310 315 320
 Ile Phe Ser Leu Phe Thr Ser Ile Tyr Arg Asn Thr Ser Phe Tyr Asn
 325 330 335
 Thr Phe Gly Phe Phe Leu Glu Lys Ser Thr Gly Ser Ser Phe Val Asp
 340 345 350
 Pro Val Ile Thr Lys Glu Phe Pro Ile Ile Ile Gly Phe Met Leu Phe
 355 360 365
 Asn Asp Leu Leu Thr Pro Leu Glu Cys Ala Met Gln Phe Val Met Ser
 370 375 380
 Leu Ile Ser Arg Thr His Glu Tyr Gln Ala Asp Ala Tyr Ala Lys Lys
 385 390 395 400
 Leu Gly Tyr Lys Gln Asn Leu Cys Arg Ala Leu Ile Asp Leu Gln Ile
 405 410 415
 Lys Asn Leu Ser Thr Met Asn Val Asp Pro Leu Tyr Ser Ser Tyr His
 420 425 430
 Tyr Ser His Pro Thr Leu Ala Glu Arg Leu Thr Ala Leu Asp Tyr Val
 435 440 445

Ser Glu Lys
450

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 315 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..315
- (D) OTHER INFORMATION: /note= "The sequence of the Rcelp protein from yeast presented as a polypeptide sequence."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Leu	Gln	Phe	Ser	Thr	Phe	Leu	Val	Leu	Leu	Tyr	Ile	Ser	Ile	Ser	
1				5					10					15		
Tyr	Val	Leu	Pro	Leu	Tyr	Ala	Thr	Ser	Gln	Pro	Glu	Gln	Ser	Lys	Arg	
			20					25					30			
Asp	Asn	Pro	Arg	Thr	Ile	Lys	Ser	Arg	Met	Gln	Lys	Leu	Thr	Ile	Met	
		35					40					45				
Leu	Ile	Ser	Asn	Leu	Phe	Leu	Val	Pro	Phe	Leu	Gln	Ser	Gln	Leu	Ser	
	50				55					60						
Ser	Thr	Thr	Ser	His	Ile	Gly	Phe	Lys	Asp	Ala	Phe	Leu	Gly	Leu	Gly	
65					70				75					80		
Ile	Ile	Pro	Gly	Tyr	Tyr	Ala	Ala	Leu	Pro	Asn	Pro	Trp	Gln	Phe	Ser	
			85						90					95		
Gln	Phe	Val	Lys	Asp	Leu	Thr	Lys	Cys	Val	Ala	Met	Leu	Leu	Thr	Leu	
			100					105					110			
Tyr	Cys	Gly	Pro	Val	Leu	Asp	Phe	Val	Leu	Tyr	His	Leu	Leu	Asn	Pro	
		115					120					125				
Lys	Ser	Ser	Ile	Leu	Glu	Asp	Phe	Tyr	His	Glu	Phe	Leu	Asn	Ile	Trp	
		130				135					140					
Ser	Phe	Arg	Asn	Phe	Ile	Phe	Ala	Pro	Ile	Thr	Glu	Glu	Ile	Phe	Tyr	
145				150					155					160		
Thr	Ser	Met	Leu	Leu	Thr	Thr	Tyr	Leu	Asn	Leu	Ile	Pro	His	Ser	Gln	
			165					170					175			
Leu	Ser	Tyr	Gln	Gln	Leu	Phe	Trp	Gln	Pro	Ser	Leu	Phe	Phe	Gly	Leu	
			180					185					190			
Ala	His	Ala	His	His	Ala	Tyr	Glu	Gln	Leu	Gln	Glu	Gly	Ser	Met	Thr	
		195				200					205					
Thr	Val	Ser	Ile	Leu	Leu	Thr	Thr	Cys	Phe	Gln	Ile	Leu	Tyr	Thr	Thr	
	210					215					220					

Leu 225	Phe	Gly	Gly	Leu 230	Thr	Lys	Phe	Val	Phe	Val 235	Arg	Thr	Gly	Gly	Asn 240
Leu	Trp	Cys	Cys	Ile 245	Ile	Leu	His	Ala	Leu 250	Cys	Asn	Ile	Met	Gly 255	Phe
Pro	Gly	Pro	Ser 260	Phe	Leu	Asn	Leu	His 265	Phe	Thr	Val	Val	Asp 270	Lys	Lys
Ala	Gly	Arg 275	Ile	Ser	Lys	Leu	Val 280	Ser	Ile	Trp	Asn	Lys 285	Cys	Tyr	Phe
Ala 290	Leu	Leu	Val	Leu	Gly	Leu 295	Ile	Ser	Leu	Lys	Asp 300	Thr	Leu	Gly	Thr
Leu 305	Val	Gly	Thr	Pro	Gly 310	Tyr	Arg	Ile	Thr	Leu 315					

WHAT IS CLAIMED IS:

1. A method for inhibiting the proteolytic removal of an AAX tripeptide from a prenylated CAAX protein in a cell, comprising introducing a mutation
5 into a gene selected from the group consisting of *AFC1*, and *RCE1*.

2. The method of claim 1, wherein the prenylated CAAX protein is selected from the group consisting of Ras protein, a-factor, and the γ -subunit of heterotrimeric G-protein.
10

3. A method for inhibiting the proteolytic removal of an AAX tripeptide from a prenylated CAAX protein in a cell, comprising blocking the activity of a second protein selected from the group consisting of Afc1p, and Rce1p.
15

4. The method of claim 3, wherein the method further comprises contacting the second protein with an inhibitor selected from the group consisting of 1,10-phenantroline and NME 181.
20

5. A method of identifying a compound which inhibits the proteolytic removal of an AAX tripeptide of a CAAX protein in a cell comprising:
providing a test compound;
contacting the test compound to a cell expressing both an *AFC1*, and an *RCE1* gene; and,
measuring an activity selected from the group consisting of Afc1p activity, Rce1p activity, Afc1p expression, and Rce1p expression.
25

6. The method of claim 5, wherein activity is measured by monitoring heat shock sensitivity of the cell.

7. The method of claim 5, wherein expression is measured using an ELISA.
30

WHAT IS CLAIMED IS:

1. A method for inhibiting the proteolytic removal of an AAX tripeptide from a prenylated CAAX protein in a cell, comprising introducing a mutation
5 into a gene selected from the group consisting of *AFC1*, and *RCE1*.
2. The method of claim 1, wherein the prenylated CAAX protein is selected from the group consisting of Ras protein, a-factor, and the γ -subunit of heterotrimeric G-protein.
10
3. A method for inhibiting the proteolytic removal of an AAX tripeptide from a prenylated CAAX protein in a cell, comprising blocking the activity of a second protein selected from the group consisting of Afc1p, and Rce1p.
4. The method of claim 3, wherein the method further comprises
15 contacting the second protein with an inhibitor selected from the group consisting of 1,10-phenantroline and NME 181.
5. A method of identifying a compound which inhibits the proteolytic
20 removal of an AAX tripeptide of a CAAX protein in a cell comprising:
providing a test compound;
contacting the test compound to a cell expressing both an *AFC1*, and an *RCE1* gene; and,
measuring an activity selected from the group consisting of Afc1p activity, Rce1p
25 activity, Afc1p expression, and Rce1p expression.
6. The method of claim 5, wherein activity is measured by monitoring heat shock sensitivity of the cell.
7. The method of claim 5, wherein expression is measured using an
30 ELISA.

8. A method of detecting heat shock sensitivity, comprising:
providing a plurality of aliquoted yeast strains in liquid;
separating each strain into a test population of cells and a control population of cells;
5 heating the test population of cells to a heat shock temperature of between about 40°C and about 60°C for a time period of between about 30 seconds and about 10 minutes, followed by cooling to a temperature of between about 0°C and about 35°C;
growing the test population of cells and the control population of cells on growth media;
10 quantitating the number of cells which grow on the growth media; and,
comparing the number of cells in the test population and the control population, whereby the comparison of the number of cells in the test population and the control population provides a measure of heat shock sensitivity.
- 15 9. The method of claim 8, wherein the yeast strains are $\Delta afc1$, or $\Delta rce1$, or both $\Delta afc1$ and $\Delta rce1$.
- 20 10. A vector comprising a nucleic acid sequence which encodes a polypeptide selected from the group consisting of Afc1p, Rce1p, and conservatively modified variations thereof, wherein the nucleic acid is operably linked to a promoter.
- 25 11. The vector of claim 10, wherein the nucleic acid sequence is selected from the group consisting of the sequences of SEQ ID NO:1 and SEQ ID NO:2.
12. A vector comprising a nucleic acid sequence which hybridizes under stringent conditions to a nucleic acid selected from the group consisting of *AFC1*, *RCE1*, and conservatively modified variations thereof.

13. The vector of claim 12, wherein the nucleic acid sequence is selected from the group consisting of the sequences of SEQ ID NO:1 and SEQ ID NO:2.
- 5 14. An isolated polypeptide encoded by the vector of claim 12.
15. The polypeptide of claim 14, wherein the polypeptide is selected from the group consisting of the sequences of SEQ ID NO:3 and SEQ ID NO:4.
- 10 16. An antibody which specifically binds to the isolated polypeptide of claim 14.
17. A recombinant cell transduced with the vector of claim 12.
- 15 18. The recombinant cell of claim 17, wherein the cell is a yeast cell.
19. The recombinant cell of claim 17, wherein the vector encodes a nucleic acid selected from the group consisting of the sequences of SEQ ID NO:1 and SEQ ID NO:2.
- 20 20. A kit for the detection of *AFCI* activity comprising a container and a reagent selected from the group consisting of a nucleic acid which hybridizes to SEQ ID NO:1, and an antibody which binds Afc1p.
- 25 21. A kit for the detection of *RCEI* activity comprising a container and a reagent selected from the group consisting of a nucleic acid which hybridizes to SEQ ID NO:2, and an antibody which binds Rce1p.

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

AFC1 AND RCE1: ISOPRENYLATED CAAX PROCESSING ENZYMES

and for which a patent application:

☒ is attached hereto and includes amendment(s) filed on _____ (if applicable)
☐ was filed in the United States on 06/023,491 as Application No. 06/023,491 (for declaration not accompanying application)
with amendment(s) filed on 06/023,491 (if applicable)
☐ was filed as PCT international Application No. 06/023,491 on 06/023,491 and was amended under PCT Article 19 on 06/023,491 (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE
60/023,491	August 7, 1996

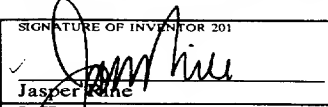
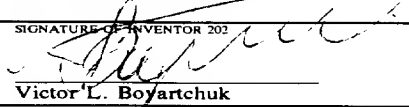
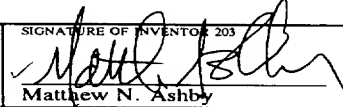
I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint S. Leslie Misrock (Reg. No. 18872), Harry C. Jones, III (Reg. No. 20280), Berj A. Terzian (Reg. No. 20060), Gerald J. Flintoft (Reg. No. 20823), David Weild, III (Reg. No. 21094), Jonathan A. Marshall (Reg. No. 24614), Barry D. Rein (Reg. No. 22411), Stanton T. Lawrence, III (Reg. No. 25736), Isaac Jarkovsky (Reg. No. 22713), Joseph V. Colaiaanni (Reg. No. 20019), Charles E. McKenney (Reg. No. 22795), Philip T. Shannon (Reg. No. 24278), Francis E. Morris (Reg. No. 24615), Charles E. Miller (Reg. No. 24576), Gidon D. Stern (Reg. No. 27469), John J. Lauter, Jr. (Reg. No. 27814), Brian M. Poissant (Reg. No. 28462), Brian D. Coggio (Reg. No. 27624), Rory J. Radding (Reg. No. 28749), Stephen J. Harbulak (Reg. No. 29166), Donald J. Goodell (Reg. No. 19766), James N. Palik (Reg. No. 25510), Thomas E. Friebe (Reg. No. 29258), Laura A. Coruzzi (Reg. No. 30742), Jennifer Gordon (Reg. No. 30753), Jon R. Stark (Reg. No. 30111), Allan A. Fanucci (Reg. No. 30256), Geraldine F. Baldwin (Reg. No. 31232), Victor N. Balancia (Reg. No. 31231), Albert P. Halluin (Reg. No. 25227), Samuel B. Abrams (Reg. No. 30605), Steven I. Wallach (Reg. No. 35402), Marcia H. Sundeen (Reg. No. 30893), Paul J. Zegger (Reg. No. 33821), Edmond R. Bannon (Reg. No. 32110), Bruce J. Barker (Reg. No. 33291), Adriane M. Antler (Reg. No. 32605), Ann L. Gisolfi (Reg. No. 31956), SaraLynn Mandel (Reg. No. 31853), Mark A. Farley (Reg. No. 33170), James G. Markey (Reg. No. 31636), and Charles F. Hoyng (Reg. No. 35548), all of Pennie & Edmonds LLP, whose addresses are 1155 Avenue of the Americas, New York, New York 10036, 1667 K Street N.W., Washington, DC 20006 and 3300 Hillview Avenue, Palo Alto, CA 94304-1203, and each of them, my attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith.

SEND CORRESPONDENCE TO: PENNIE & EDMONDS LLP 1155 AVENUE OF THE AMERICAS NEW YORK, N.Y. 10036-2711				DIRECT TELEPHONE CALLS TO: PENNIE & EDMONDS LLP DOCKETING (212) 790-2803	
201	FULL NAME OF INVENTOR	LAST NAME Rine	FIRST NAME Jasper	MIDDLE NAME D.	
	RESIDENCE & CITIZENSHIP	CITY Moraga	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States of America	
	POST OFFICE ADDRESS	STREET 3878 Paseo Grande	CITY Moraga	STATE OR COUNTRY California	ZIP CODE 94556
202	FULL NAME OF INVENTOR	LAST NAME Boyartchuk	FIRST NAME Victor	MIDDLE NAME L.	
	RESIDENCE & CITIZENSHIP	CITY Berkeley	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP Ukraine	
	POST OFFICE ADDRESS	STREET 1741 Martin Luther King Jr. Way, #6A	CITY Berkeley	STATE OR COUNTRY California	ZIP CODE 94709
203	FULL NAME OF INVENTOR	LAST NAME Ashby	FIRST NAME Matthew	MIDDLE NAME N.	
	RESIDENCE & CITIZENSHIP	CITY Mill Valley	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States of America	
	POST OFFICE ADDRESS	STREET 91 Longfellow Road	CITY Mill Valley	STATE OR COUNTRY California	ZIP CODE 94941
204	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
205	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
206	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 	SIGNATURE OF INVENTOR 202 	SIGNATURE OF INVENTOR 203 
Jasper Rine	Victor L. Boyartchuk	Matthew N. Ashby
DATE 7-16-97	DATE 7-16-97	DATE 7-22-97
SIGNATURE OF INVENTOR 204	SIGNATURE OF INVENTOR 205	SIGNATURE OF INVENTOR 206
DATE	DATE	DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Jasper D. Rine et al. Art Unit:
Serial No.: unknown Examiner:
Filed : 10/2/98
Title : AFC1 AND RCE1: ISOPRENYLATED CAAX PROCESSING ENZYMES

Assistant Commissioner for Patents
Washington, DC 20231

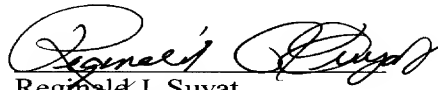
ASSOCIATE POWER OF ATTORNEY

Please recognize Lisa A. Haile, Reg. No. 38,347; Christopher P. Rogers, Reg. No. 36,334, and Susan A. Siegel, Reg. No. 43,121, as my associate attorneys/agents in the above application, with full powers.

Please continue to direct all official communications to the undersigned.

Respectfully submitted,

Date: October 2, 1998

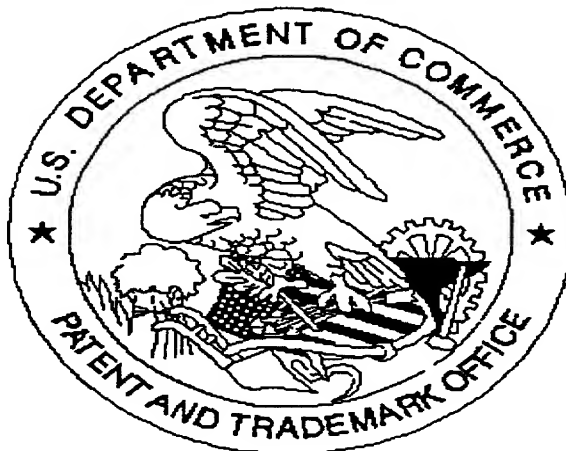

Reginald J. Suyat
Reg. No. 28,172

Fish & Richardson P.C.
2200 Sand Hill Road, Suite 100
Menlo Park, CA 94025

Telephone: 650/322-5070
Facsimile: 650/854-0875

98034 PAL1

United States Patent & Trademark Office
Office of Initial Patent Examination -- Scanning Division



Application deficiencies were found during scanning:

☐ Page(s) _____ of DRAWINGS. were not present
for scanning. (Document title)

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Scanned copy is best available.